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Atrial Natriuretic Peptide: Synthesis, Release, and Metabolism*

HEIKKI RUSKOAHO

Department of Pharmacology and Toxicology, University of Oulu, Oulu, Finland

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I. Introduction

ANP† is a cardiac hormone that is secreted primarily by atrial myocytes in response to local wall stretch. ANP elicits a number of actions including (*a*) a decrease in blood pressure (in part mediated by direct relaxation of vascular smooth muscle); (*b*) an increase in salt and water excretion; (*c*) a facilitation of transudation of plasma water to the interstitium; and (*d*) an inhibition of the release or actions of several hormones, such as aldosterone, ANG II, ET, renin, and vasopressin. The actions of ANP on vasculature, kidneys, adrenals, and other organs serve both acutely and chronically to reduce systemic blood pressure as well as intravascular volume (fig. 1). Specific receptors that bind ANP with high affinity have been identified in a variety of tissues, and evidence has been presented that its cellular action involves increased formation of cGMP because of activation of a particulate guanylate cyclase. Because of its

† Abbreviations: ANP, atrial natriuretic peptide; ACE, angiotensin-converting enzyme; ANG, angiotensin; ANF, atrial natriuretic factor; AVP, arg^8-vasopressin; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; cAMP, cyclic adenosine 3′,5′-monophosphate; cGMP, cyclic guanosine 3′,5′-monophosphate; CHF, congestive heart failure; DAG, diacylglycerol; DCM, dilated cardiomyopathy; EDRF, endothelium-derived relaxant factor; ET, endothelin; GFR, glomerul filtration rate; IP_3, inositol trisphosphate; IR-ANP, immunoreactive ANP; IR-BNP, immunoreactive BNP; IR-NT-ANP, immunoreactive NT-ANP; NT-ANP, NH_2-terminal ANP; PKC, protein kinase C; SHR, spontaneously hypertensive rat(s); TPA, 12-O-tetradecanoyl-phorbol 13-acetate; WKY, Wistar-Kyoto; AP, activator protein; AV3V, anteroventral region of the third ventricle; CAT, chloramphenicol acetyltransferase; bp, base pair; kb, kilobase; SV40, simian virus 40; PG, prostaglandins; DOCA, desoxycorticosterone acetate; Dahl-S, Dahl salt-sensitive strain; Dahl-R, Dahl salt-resistant atrain; DI, diabetes insipidus strain; SHR-SP, SHR-stroke-prone strain; $t_{1/2}$, half-life; T_4, thyroxine; CGRP, calcitonin gene-related peptide; i.v., intravenous(ly); i.c.v., intracerebroventricular(ly); NEP, neutral endopeptidase; V_{dss}, volume of distribution.

unique combination of actions, ANP is undergoing extensive evaluation as a hypotensive and diuretic agent for the treatment of CHF, hypertension, renal failure, and states of fluid overload. In addition, the measurement of plasma IR-ANP concentrations may serve as a useful noninvasive marker for cardiac overload. Recent advances in knowledge of the synthesis, secretion, and removal of ANP from the circulation are summarized and integrated in this review.

II. Structure, Synthesis, and Processing of Atrial Natriuretic Peptide

A. Structure of Atrial Natriuretic Peptide

Kisch (1956) first described granules in the atrial myocytes, and by 1964 similar granules had been described in a number of mammalian species (Jamieson and Palade, 1964). These granules were suggested to be formed in the large Golgi complex characteristic of atrial cardiocytes. Smaller species (rat, mouse) generally have larger and more numerous granules than larger animals (dog, cat, human) (Jamieson and Palade, 1964). Rat atriocytes may contain as many as 600 granules per cell, ranging in diameter from 0.2 to 0.5 μm (average, 0.42 μm) with more granules in the right than in the left atrium. Younger animals have fewer granules per cell than older animals. Jamieson and Palade (1964) also reported that these granules were present in all mammalian atriocytes but not in ventricular myocytes. Morphologically and histochemically, the granules resemble the secretory granules found in peptide-secreting cells (Huet and Cantin, 1974; de Bold and Bencosme, 1975).

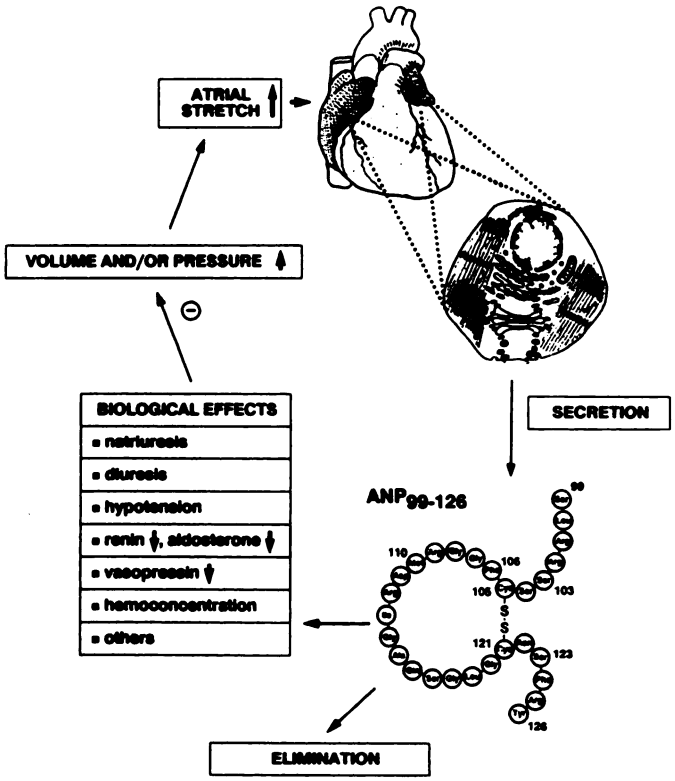

FIG. 1. ANP hormonal system.

Changes in dietary sodium and water have been shown to alter the granularity of rat atrial myocytes, suggesting that the atria may be involved in the control of extracellular fluid volume (Marie et al., 1976; de Bold, 1979). However, the content and function of these granules remained unclear until de Bold et al. (1981) demonstrated that i.v. administration of rat atrial (but not ventricular) extracts into intact rats produces a profound natriuresis and diuresis. They suggested that the granules contained a natriuretic substance which they named ANF. This discovery led to the prompt identification of the molecular structure of circulating ANP and its precursors in atrial tissue. The hormone initially had many names, including atrial natriuretic hormone, atrial natriuretic substance, atriopeptin, atrin, auriculin, cardiodilatin, or cardionatrin. Further details of the history and discovery of ANP may be found in earlier reviews (de Bold, 1985; Cantin and Genest, 1985; Flynn and Davies, 1985; Laragh, 1985; Maack et al., 1985; Needleman et al., 1985; Ballermann and Brenner, 1986; Forssmann et al., 1986b; Needleman and Greenwald, 1986; de Bold, 1987; Fried, 1987; Lang et al., 1987; Genest and Cantin, 1988; Goetz, 1988; Needleman et al., 1989).

The ANPs are derived from a common precursor, called preproANP, which contains between 149 and 153 amino acids depending upon the species (fig. 2). The human sequence, deduced by cloning and characterizing cDNAs from mRNA, consists of 151 amino acids (Nakayama et al., 1984; Oikawa et al., 1984; Zivin et al., 1984) and shares strong homology with peptides from rat (152 amino acids) (Kangawa et al., 1984c; Maki et al. 1984b; Seidman et al., 1984b, Yamanaka et al., 1984; Zivin et al., 1984; Flynn et al., 1985), mouse (Seidman et al., 1984a), dog (Oikawa et al., 1985), rabbit (Oikawa et al., 1985), and pig (Mägert et al., 1990). Each preproANP molecule contains a signal peptide sequence at its amino-terminal end. ANP is mainly stored as a 126-amino acid peptide, proANP$_{1-126}$ (also called proANP in this review) (Thibault et al., 1987), which is produced by cleavage of the signal peptide. When appropriate signals for hormone release are given, proANP$_{1-126}$ is further split into an NH_2-terminal fragment, proANP$_{1-98}$ (Michener et al., 1986), and the biologically active hormone, the COOH-terminal peptide, ANP$_{99-126}$ (or ANP$_{1-28}$) (Schwartz et al., 1985; Thibault et al., 1985).

The amino acid sequence of the precursor molecule proANP$_{1-126}$ is highly homologous (approximately 74%) in humans, rat, mouse, dog, and rabbit (Oikawa et al., 1985). The COOH-terminal 45-residue sequence, particularly, is remarkably consistent with 93% homology in the five species. With the exception of residue 110 (12), the amino acid sequences of the biologically active ANP$_{99-126}$ (ANP$_{1-28}$) hormone are identical in all mammalian species. The amino acid at residue 110 is methi-

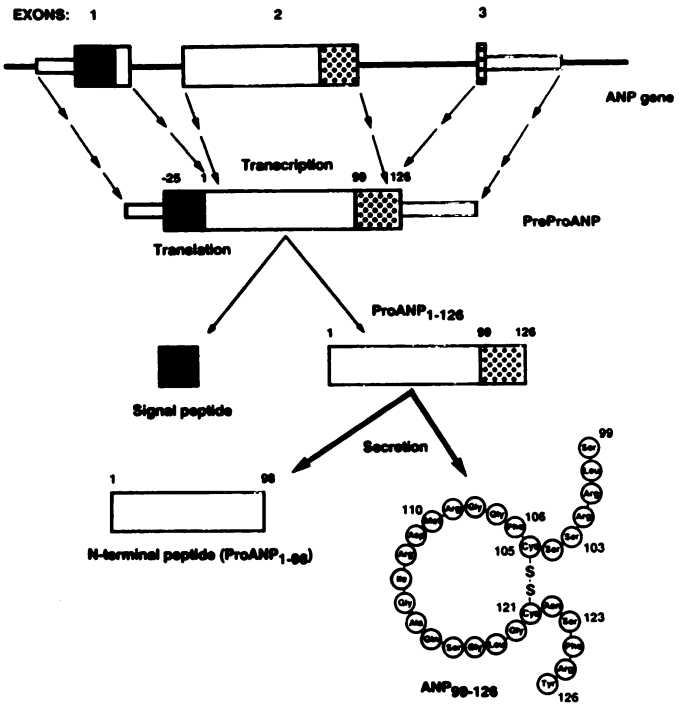

FIG. 2. Processing pathways and major molecular forms of ANP.

onine in humans (Kangawa and Matsuo, 1984), pigs (Forssmann et al., 1984), dogs (Oikawa et al., 1985), cows (Ong et al., 1986; Vlasuk et al., 1986), and sheep (Yandle et al., 1991) but isoleucine in rats (Flynn et al., 1983), mice (Seidman et al., 1984a), and rabbits (Wei et al., 1986). Although mammalian ANPs are highly similar in structure, nonmammalian amino acid sequences differ considerably from their mammalian counterparts. Maximum homology between the nonmammalian ANPs such as frog (fANP-21, fANP-24, fANP-30) (Lazure et al., 1988; Sakata et al., 1988a), eel (eANP-27) (Takei et al., 1989), and mammalian ANP is seen in the 17-amino acid ring structure, whereas unique features different from those of mammalian ANP are noted both in NH_2- and COOH-terminal regions of nonmammalian ANP (Lazure et al., 1988; Sakata et al., 1988a; Takei et al., 1989).

Many molecular and biochemical features of ANP that are important for the physiological effects of all natriuretic peptide hormones have been identified (for reviews, see Nutt and Veber, 1987; Genest and Cantin, 1988; Bovy, 1990). Active ANP analogs share a common central ring structure formed by a disulfide bridge between cysteine residues at positions 105 (7) and 121 (23). This structure is preserved in all members of the natriuretic peptide hormone family, and the disruption of the ring by hydrolytic cleavage leads to loss of biological activity (Atlas et al., 1984; Chartier et al., 1984; Misono et al., 1984). Studies of structure-activity relationships have also shown the importance of the COOH-terminal residues; deletion of amino acids from the COOH terminus progressively reduces the natriuretic and vasorelaxant potency of ANP analogs (Nutt and Veber, 1987; Genest and Cantin, 1988; Bovy, 1990). Extensions or deletions at ANP's amino-terminal end influence the hormone's bioactivity less critically, with proANP reported to have vasorelaxant, natriuretic and hypotensive properties (Currie et al., 1984a; Forssmann et al., 1984; Flynn et al., 1985; Kangawa et al., 1985). However, proANP's bioactivity after administration in vivo may be due to a conversion to the ANP_{99-126}.

B. Cardiac Biosynthesis

In the normal adult mammalian heart, ANP is synthesized predominantly in the cardiac atriocytes, where ANP mRNA levels make up 1 to 3% of all mRNA species (Seidman et al., 1984b; Yamanaka et al., 1984). Small quantities of ANP mRNA have been detected in normal adult ventriculocytes, where levels in the left ventricle may be approximately 0.5 to 3% of atrial concentrations (Zivin et al., 1984; Gardner et al., 1986a; Nemer et al., 1986; Lattion et al., 1986; Arai et al., 1988; Ogawa et al., 1991), but higher levels are induced by cardiac overload and hormones (Gardner et al., 1986b; Lattion et al., 1986; Day et al., 1987; Dene and Rapp, 1987; Takayanagi et al., 1987). In addition, a number of extracardiac tissues, including the central nervous system, lung, adrenal gland, kidney, and vascular tissue have also been shown to express detectable levels of the ANP gene product (<1% of those of atria) (for reviews, see Forssmann et al., 1989; Gutkowska and Nemer, 1989; Vollmar, 1990).

1. Developmental regulation. Expression of ANP is developmentally regulated in cardiac atriocytes and ventriculocytes (Bloch et al., 1986; Cantin et al., 1987, Glembotski et al., 1987; Kikuchi et al., 1987; Wei et al., 1987b; Zeller et al., 1987). Atrial ANP mRNA is initially low and increases through development. In contrast, the developing cardiac ventriculocytes contain substantial ANP mRNA that rapidly declines after birth. In the mouse, ANP mRNA was first detected in a subpopulation of myocardial cells at day 8 of embryogenesis using in situ hybridization to histological sections (Zeller et al., 1987). On day 9, abundant hybridization of the ANP probe to the atrium and the primitive ventricle was found, and by day 14, strong labeling of cells in both atria and the trabeculated regions of the left ventricle was seen; labeling in the ventricle was comparable to hybridization of the atria (Zeller et al., 1987). In the fetus and neonate, as in the adult, virtually all of the ventricular ANP mRNA is expressed in ventriculocytes in the left ventricle (Wei et al., 1987b; Zeller et al., 1987). The ANP mRNA levels in the rat ventriculocyte during fetal and neonatal life peak on days 1 to 2 after birth (Bloch et al., 1986; Wei et al., 1987b; Wu et al., 1988). During development, some species difference is seen in the rate of decline in left ventricular ANP mRNA. In rats 2 weeks postpartum, ANP mRNA levels in the left ventricle are still higher than in the adult left ventricle (Wei et al., 1987b), whereas in 1-day-old mice, ANP mRNA levels

in the left ventricle are similar to adult levels (Zeller et al., 1987). In 18-day-old embryonic chick hearts, ANP mRNA is predominantly expressed in the left and right ventricles (Chernin et al. 1990). The early appearance and developmental regulation of ANP gene expression in atrial and ventricular cells suggest an important role for this peptide hormone in the embryo.

The changes in atrial and ventricular ANP mRNA levels parallel the changes in fetal and neonatal content of IR-ANP (Dolan and Dobrozsi, 1987; Snajdar et al., 1987; Wei et al., 1987b; Wu et al., 1988; Navaratnam et al., 1989). The content of IR-ANP in the developing human heart, as determined by radioimmunoassays, is greatest in left atrial cells and occurs decreasingly in right atriocytes, right ventriculocytes, and left ventriculocytes (Kikuchi et al., 1987). The IR-ANP concentration in the ventricle is >1 μg/g protein in human fetuses at 14 and 22 weeks' gestation and decreases as the fetus develops (Kikuchi et al., 1987). In rats, atrial IR-ANP concentrations are at least 10-fold greater than those found in the ventricle (Wei et al., 1987b; Wu et al., 1988).

IR-ANP granules have been demonstrated by immunohistochemical techniques in both atrial and ventricular cardiocytes in fetal and newborn rats, humans, and hamsters (Bloch et al., 1986; Thompson et al., 1986; Scott and Jennes, 1987; Toshimori et al., 1987a; Gilloteaux, 1989; Navaratnam et al., 1989; Larsen, 1990; Hassall et al., 1990). These immunoreactive cells concentrate in the atria as maturation progresses but are virtually absent in ventricular cardiocytes of adult mammalian species (Chapeau et al., 1985; McKenzie et al., 1985). These findings extend the early ultrastructural studies of the developing and adult mammalian heart in which secretory granules, whose function was unknown, were noted within the atriocytes and ventriculocytes of the embryonic heart (for further references, see Thompson et al., 1986). Because the fetal heart also responds to exogenous stimuli (Wei et al., 1987b; for reviews, see Smith et al., 1989; Semmelkrot and Guignard, 1991) during perinatal and postnatal development, as do adult animals, the entire heart appears to have endocrine function.

It is not clear which factors regulate the developmental tissue-specific expression of ANP. One possibility, as suggested by Zeller et al. (1987), is that certain cells are committed for ANP expression early during development. Alternatively, the changes in atrial and ventricular ANP expression during fetal life and after birth may reflect marked changes in intracardiac pressure, vascular resistance, and hormone levels (see section III.F). Thus, although the molecular determinants responsible for the tissue-specific expression and developmental regulation have not yet been fully elucidated, the ANP gene serves as a useful model for the study of cardiac specific gene expression. Expression of the ANP also represents a useful marker of cardiac myogenesis, because transcriptional activation of the ANP gene in culture was a consistent phenotypic marker of differentiating cardiac myoblasts, regardless of their anatomical location (Kohtz et al., 1989).

2. Atrial and ventricular expression of atrial natriuretic peptide in the normal adult heart. ANP is present in high concentrations in the atria of several species, as shown by radioimmunoassays and ANP mRNA measurements (for review, see Genest and Cantin, 1988). Adult rat atriocytes contain about 100- to 150-fold more ANP mRNA than ventricular myocytes (Nemer et al., 1986). In situ hybridization studies have confirmed ANP gene expression in the atria and ventricles (Nemer et al., 1986; Lattion et al., 1986; Glembotski et al., 1987; Hamid et al., 1987; Sutin et al., 1990). Secretory ANP granules have been visualized by immunohistochemical methods within the atriocytes, predominantly located in a perinuclear position (Cantin et al., 1984; Forssmann et al., 1984). ANP mRNA and ANP peptides are present in cells in both the right and left atria as well as in the atrial appendage. IR-ANP concentrations are generally higher in the right than in the left atrium in normal adult animals, such as the rat (Cantin et al., 1984; Gutkowska et al., 1984b; Tanaka et al., 1984; Tang et al., 1984a; Chapeau et al., 1985), although there are exceptions, e.g., the rabbit, dog, and frog hearts, in which higher concentrations of IR-ANP have been reported in the left than in right atrial cells (Netchitailo et al., 1986; Cernacek et al., 1988a; Synhorst and Gutkowska, 1988). In the normal adult rat, each atrium contains 5 to 10 μg IR-ANP (Gutkowska et al., 1984b; Nakao et al., 1984; Cantin et al., 1985).

Whereas adult rat atriocytes contain 100 to 150 times as much ANP mRNA as ventriculocytes, they contain a 1000-fold higher concentration of IR-ANP per mg of tissue (Nemer et al., 1986) with concentrations in ventricular cells of 0.0036 μg IR-ANP/mg of tissue compared with 0.3 μg/mg in atriocytes. IR-ANP and ANP mRNA in normal human ventricular tissue are detectable by radioimmunoassays and Northern blots, respectively (Saito et al., 1987b; Tsuchimochi et al., 1988; Saito et al., 1989a), although in small quantities. Thus, measurement of steady-state levels of mRNA in endomyocardial biopsies using polymerase chain reaction failed to detect ANP mRNA in the nonfailing human heart (Feldman et al., 1991). The IR-ANP level in the adult normal ventriculocyte is about 15 to 20 ng/g (ranging from 4 to 40 ng/g depending on the part of the ventricle), this level being only 0.04% of the atrial level (Kikuchi et al., 1987; Saito et al., 1989a). The total content of IR-ANP in the ventricle was 0.26% of that in the atrium, and the total content of ANP mRNA level was 0.22% of that in the atria (Saito et al., 1987b, 1989a). The simultaneous detection of ANP mRNA and IR-ANP in the rat and human normal ventricle shows that ANP is synthesized in the adult ventriculocyte as well as in the human

atriocyte. Although low, the ventricular ANP level is one of the highest ANP levels in extraatrial tissues including the brain (Gutkowska and Nemer, 1989; Vollmar, 1990).

Little or no ANP granules have been found by immunohistochemical methods in any ventricular sections obtained from normal human hearts (Tsuchimochi et al., 1987; Edwards et al., 1988a; Skepper et al., 1988; Truong et al., 1989; Arbustini et al., 1990) or experimental animals (Cantin et al., 1984; Forssmann et al., 1984; Metz et al., 1984; Tang et al., 1984a; Chapeau et al., 1985; McKenzie et al., 1985; Vuolteenaho et al., 1985a; Rinne et al., 1986; Toshimori et al., 1987b; Toshimori et al., 1988c; Navaratnam et al., 1989), probably because small amounts of peptide are likely to escape immunohistochemical detection. However, ANP immunoreactivity is present in the ventricular impulse-conducting system or associated cells by immunohistochemistry (Back et al., 1986; Toshimori et al., 1987b; Wharton et al., 1988a; Cantin et al., 1989; Forssmann et al., 1989; Jougasaki et al., 1989; Skepper, 1989). The position of the ANP cells in the trabeculae of the fetal rat and mouse hearts correlates with the position of the developing Purkinje fibers (Thompson et al., 1986; Scott and Jennes, 1987). Rabbit Purkinje fibers contain IR-ANP in their secretory granules and their Golgi complex and exhibit ANP mRNA, as visualized by in situ hybridization (Anand-Srivastava et al., 1989). Purkinje fibers contain levels of IR-ANP and ANP mRNA intermediate between those of working atrial and ventricular cardiocytes (Anand-Srivastava et al., 1989; Mochizuki et al., 1991). The sinus nodal cells and the transitional cells that surround them also showed ANP immunoreactivity (Skepper, 1989; Sola et al., 1990), and their ANP gene expression measured by in situ hybridization was well above the level of that found in normal ventricles (Sola et al., 1990). The role of ANP in the impulse-conducting system is not known, but ANP may have both autocrine and paracrine function in the modulation of the impulse in the peripheral conduction cells.

ANP-like substances have been detected in the hearts of various nonmammalian species mostly through the use of immunological methods such as radioimmunoassay and immunohistochemistry. Ventricular cardiocytes of nonmammalian species such as amphibians, reptiles, and birds have been shown to contain specific granules (Bencosme and Berger, 1971; Yunge et al., 1980) and ANP bioactivity (de Bold and Salerno, 1983). Antibodies directed against the bioactive core produced a positive reaction in the atria of toad, frog, snake, hen, and fish (Chapeau et al., 1985). Similar results were described by Reinecke et al. (1985), who showed that cardiac hormones can be found in avian, reptilian, amphibian, and bony fishes' hearts. ANP-like peptides together with the precursor forms were described in both frog atria and ventricles (Netchitailo et al., 1986, 1988), and atrial and ventricular cells of fish secrete IR-ANP when maintained in tissue culture (Baranowski and Westenfelder, 1989). Yet, ANP immunoreactivity is limited to a cardiac neurosecretory system in the snail, and cardiac atriocytes and ventriculocytes do not contain IR-ANP (Nehls et al., 1985). Thus a relocation of ANP gene activity has occurred during evolution. A gene activity in lower nonvertebrates, such as snail, is confined to neuronal tissue; in lower vertebrates, such as reptiles and amphibians, it is found in both atrial and ventricular myocytes; and in higher mammals, including humans, ANP gene expression is demonstrable mainly in atria. However, in disease states such as heart failure, ANP gene expression can also be seen in ventricular myocytes, demonstrating phylogenetic regression.

Results of recent studies suggest that even single-cell organisms and plants contain the ANP-like hormonal system. The NH_2- and COOH-terminal ANP-like peptides, measured by radioimmunoassays using antibodies that recognize human sequences, were found within *Paramecium multimicronucleatum* at concentrations similar to their respective concentrations in the rat plasma (Vesely and Giordano, 1992). Interestingly, the ANP_{99-126} and proANP were immunologically recognized to be present also in the stems and leaves of the Florida Beauty (*Dracena godseffiana*) (Vesely and Giordano, 1991), suggesting that this plant can synthesize ANP similarly to humans and vertebrate animals. The amount of ANP-like prohormone in plant leaves and stems was quite remarkable, being approximately half of the amount found in rat atria and clearly higher than its concentration in the rat ventricle (Vesely and Giordano, 1991).

3. Structure of the atrial natriuretic peptide gene. Genomic clones containing DNA encoding the human (Greenberg et al., 1984; Maki et al., 1984a; Nemer et al., 1984; Seidman et al., 1984a), rat (Argentin et al., 1985), mouse (Seidman et al., 1984a), and cow (Vlasuk et al., 1986) preproANP peptides have been sequenced. The structural organization of the ANP gene has been highly conserved through evolution (for reviews, see Baxter et al., 1988; Rosenzweig and Seidman, 1991). A single gene has been identified in all mammalian species examined to date and contains many features of a typical eukaryotic gene. The proximal flanking sequence upstream of the transcription initiation site contains elements characteristic of eukaryotic promoters, including a TATAA box approximately 30 bp upstream of the transcription initiation site (Seidman et al., 1988). In humans the ANP gene is located on chromosome 1 (Yang-Feng et al., 1985), band p36, and it is one of a syntenic group that is present on chromosome 4 of the mouse (Yang-Feng et al., 1985; Mullins et al., 1987). There is significant homology in the organization and nucleotide sequences of ANP genes from many species. In each, the gene consists of three exons separated by two introns. Coding block 1 encodes the first 40 to 41 amino acids, including the signal peptide and the first 20 amino acid residues of

proANP$_{1-126}$. Exon 2 encodes the remainder of proANP$_{1-126}$ except for 1 (human) or 3 (rat) COOH-terminal amino acids, which are encoded by exon 3 (fig. 2).

4. Regulatory sequences of the atrial natriuretic peptide gene. The regions of maximal homology within the mouse, rat, and human ANP genes are concentrated in the DNA sequence located between −1100 bp and the transcription initiation site (Nemer et al., 1984; Seidman et al., 1984a, 1988; Wu et al., 1991), suggesting that this region may be involved in regulating ANP gene expression. To identify sequences important for ANP transcription, portions of the 5′-flanking sequences of the ANP gene have been attached to the reporter genes, CAT or luciferase. These constructs have then been transfected into neonatal atrial or ventricular cardiocytes as well as to nonmyocardial cells. The results show that regulation of the ANP gene may involve sequences that are proximal as well as distal to the promoter apparatus. At least three *cis*-acting regulatory elements are required for expression of the rat ANP gene. Seidman et al. (1988) showed that 3.4 kb of the 5′-flanking sequence from rat ANP are sufficient to direct high-level atrial specific transcription. Studies by two groups have demonstrated that there is one distal regulatory element mediating cardiac specific expression upstream of the rat ANP gene located >638 bp (positions −2427 to −638 bp) from the transcription initiation site (Seidman et al., 1988; Knowlton et al., 1991). Important proximal regulatory sequences between −638 and −137/−134 bp were also identified (Rosenzweig et al., 1991; Knowlton et al., 1991). Deletion of these proximal regulatory sequences reduced ANP gene transcription 20- to 30-fold (Seidman et al., 1988).

Transfection experiments in primary cultured neonatal rat atrial cells with ANP sequences directing CAT gene transcription have identified two proximal regulatory elements (Rosenzweig et al., 1991). These two elements lie within a 213-bp region located between −609 and −397 bp upstream of the transcription initiation site. One proximal regulatory element contains an AP-1-like binding site and is recognized by the AP-1 protein, the c-*fos*/c-*jun* protooncogene heterodimer in vitro. The second regulatory element contains a cAMP-responsive element-like recognition site (Rosenzweig et al., 1991). Using ANP luciferase fusion genes in transient assays in neonatal rat ventricular cells, Knowlton et al. (1991) identified a 315-bp fragment (positions −636 to −323 bp) that can mediate adrenergic inducible expression of a luciferase reporter gene. Analysis of the sequences within the fragment also revealed the presence of potential cAMP-responsive element- and AP-1-binding sites as well as AP-2-, Egr-1-, and CArgG-binding sites within the ANP promoter fragment (Knowlton et al., 1991). To further elucidate the regulation of rat ANP gene expression, Seidman et al. (1991) created transgenic mice carrying a construct containing 3.4 kb of the 5′-flanking sequence in front of the bacterial reporter gene CAT. Results of these in vivo studies support the earlier findings in cardiocytes: 3.4 kb upstream is sufficient to direct high-level, atrial specific, and developmentally appropriate transcription.

A steroid-responsive element has also been detected within a region on the 5′ side of the rat ANP gene (Seidman et al., 1988; Argentin et al., 1991). DNA-mediated gene transfer studies identified a glucocorticoid response element in the distal 5′-flanking sequences of the rat gene between −697 and −1029 bp (Argentin et al., 1991). In vitro DNase footprinting experiments revealed that the purified glucocorticoid receptor bound two elements, one located between −952 and −976 bp and the other between −901 and −875 bp (Argentin et al., 1991). These elements showed partial sequence homology with consensus glucocorticoid-responsive element.

Expression of the human ANP gene is also controlled by a series of positive and negative *cis*-acting regulatory elements present in the 5′-flanking sequences of the gene. LaPointe et al. (1988) and Wu et al. (1989) found that 409 bp of upstream sequence was sufficient to direct tissue-specific expression in primary cardiocyte cultures. The importance of these sequences was confirmed by Field (1988), who reported that 472 bp of human ANP 5′-flanking sequences were sufficient to confer atrial specific expression upon the SV40 large T-antigen following the introduction of the chimeric gene (*hANP-Tag*) into transgenic mice. This study further delineated that these sequences promoted atrial specific expression, because expression of the oncogene by transgenic animals produced marked right atrial hyperplasia only; the left atrium remained normal in size (Field, 1988).

Positive elements located between −1150 and −222 bp, relative to the transcription start site, appear to be responsible for the major portion of human ANP gene expression analyzed by the ability of the deletion mutants to drive CAT expression in cultured neonatal rat atrial or ventricular cells (Lapointe et al., 1988; Wu et al., 1989, 1991). Candidate *cis*-acting sequences capable of conferring the myocardial specific activity (CAT expression) would include the elements between −1150 and −410 bp and between −410 and −332 bp as well as the less specific positive element between −332 and −222 bp, which appears to be used in myocardial as well as nonmyocardial cells (Wu et al., 1991). These regions may represent the human homologues of the functional regions described above in the rat gene, and it appears that some similar *cis*-acting elements have a different structural organization between rat and human. For example, in the human gene, one short stretch (positions −241 to −235 bp) is identical with the consensus sequence for the AP-1-binding site (Wu et al., 1991), located in the rat gene about 250 bp upstream. Interestingly, the qualitative profile of human ANP CAT expression in the

ventricular cells is very similar to that in their atrial counterparts; the major difference is in the overall level of expression (Wu et al., 1991). The upstream negative regulatory or silencer element, perhaps together with the more proximally located silencer, appears to be responsible for extinguishing ANP gene expression in nonmyocardial cells (Wu et al., 1991).

In eukaryotic cells, tissue-specific gene expression is often conferred by interaction of *cis*-acting regulatory elements with tissue-specific nuclear proteins called *trans*-acting factors. A 68-bp segment of the 5′-flanking sequence (positions −409 to −333 bp) identified by Gardner's group (LaPointe et al., 1988; Wu et al., 1989) in the human gene appeared to interact with a cardiac specific nuclear protein. The nucleotide sequence of this binding site is homologous to sequences located 1200 bp from the rat ANP gene site. However, this *cis*-acting element can also direct transcription in nonmyocardial (liver) cell types (Wu et al., 1991). This DNA-binding protein may be involved in mediating the activation of human ANP gene transcription (Wu et al., 1991). Recent data further suggest the existence of other cardiac specific nuclear proteins (Argentin et al., 1991).

C. Processing of atrial natriuretic peptide

1. Atrial natriuretic peptide posttranslational processing and processing products. Regulatory peptides are initially synthesized as large protein precursors that must undergo proteolytic processing to yield the biologically active peptide (Harris, 1989; Lingappa, 1989). Commonly, the proteolytic enzymes involved in this posttranslational processing remove the signal peptide, a hydrophobic peptide located at the amino terminus of the precursor molecule. The signal peptide is involved in the cotranslational transport of the peptide across the membrane of the endoplasmic reticulum and is cleaved at single or two adjacent basic residues by endoprotease. This selective processing may play an important role in determining which peptides are released by the cell.

As we have seen, the ANP peptides are derived from a common precursor, termed preproANP, which contains 151 amino acids in humans. Like other peptide hormones, the human preproANP molecule contains a 25-amino acid signal peptide sequence at its amino terminus (Nakayama et al., 1984; Oikawa et al., 1984; Zivin et al., 1984). The signal peptide is cleaved during the transport process, producing a protein with 126 amino acids, $proANP_{1-126}$, the predominant storage form of ANP in the atria (Tanaka et al., 1984; Kangawa et al., 1984c; Nakao et al., 1984; Bloch et al., 1985; Flynn et al., 1985; Glembotski et al., 1985; Kangawa et al., 1985; Lang et al., 1985; Vuolteenaho et al., 1985b), and ventricular myocytes (Day et al., 1987; Stockmann et al., 1988). $ProANP_{1-126}$ travels uncleaved from the Golgi complex to immature atrial granules and then to mature granules (Thibault et al., 1989a; Cantin et al., 1990); these are dispatched to the cell surface for final release of the peptide to the exterior by exocytosis (Page et al., 1986; Sugawara, 1987; Agnoletti et al., 1989; Forssmann et al., 1989; Skepper et al., 1989; Newman and Severs, 1990; Gilloteaux et al., 1991). Similarly, immunohistochemical results showed that cleavage products were not detected within rat atrial myocytes (Wildey et al., 1988). In addition, purification of ANP from these granules and its identification by amino acid composition and sequencing revealed that it corresponds to proANP (Bloch et al., 1987a; Gibson et al., 1987; Thibault et al., 1987). Thus, this part of ANP processing differs from that of most other endocrine peptides, which are processed in the rough endoplasmic reticulum, Golgi apparatus, and secretory granules to be stored as bioactive peptide hormones.

During or soon after release, $proANP_{1-126}$ is cleaved into the NT-ANP, $proANP_{1-98}$, and the major biologically active hormone, the COOH-terminal peptide ANP_{99-126}, both in rats (Schwartz et al., 1985; Thibault et al., 1985) and humans (Forssmann et al., 1986a; Theiss et al., 1987; Yandle et al., 1987). Levels of the 28-amino acid circulating form of ANP_{99-126} increase in response to physiological stimuli, indicating that it represents a biologically relevant product of $proANP_{1-126}$ (Schwartz et al., 1985). Generally, the proteolytic processing of propeptides takes place on a monobasic or dibasic residue, i.e., either only one basic amino acid is located or two basic amino acids are paired at the cleavage sites (Harris, 1989). The processing of $proANP_{1-126}$ occurs at the proximal monobasic site, Arg^{98}-Ser^{99}, resulting in the production of ANP_{99-126}. Initial reports showed that peptides of varying lengths derived from the COOH-terminal end of $proANP_{1-126}$ had natriuretic activity (for reviews, see Nutt and Veber, 1987; Genest and Cantin, 1988; Bovy, 1990). These other peptides are now believed to be nonphysiological cleavage products obtained by proteolytic digestion during extraction.

In several species, including rats, mice, cows, and rabbits, but not humans or dogs, the nucleotide sequences allow the prediction of a terminal Arg-Arg sequence at the extreme COOH terminus of the prohormone (Oikawa et al., 1985; Vlasuk et al., 1986). The dibasic residues have not been detected in purified rat atrial secretory granules, suggesting that either these residues are never translated or they are removed from the COOH terminus as an early processing event. Thibault et al. (1989b) identified a variety of different molecules with antibodies directed against different parts of $proANP_{1-126}$. One of these molecules appears to be intact ANP_{1-128}. Thus, the arginine pair is suggested to be cleaved by a carboxypeptidase E (H) or enkephalin convertase, because it can be cofractionated with ANP-containing secretory granules in the rat (Lynch et al., 1988), and this enzyme appears to be common in endocrine tissues. Another early posttranslational modification is the formation of an intra-

chain disulfide bond between cysteine residues at positions 105 and 121 to form the 17-member ring structure that appears to be essential for bioactivity of ANP. Furthermore, ANP can act as a substrate for phosphorylation, and both the phosphorylated and unphosphorylated forms are found in atrial secretory granules and secreted (Rittenhouse et al., 1986; Bloch et al., 1987b; Wildey et al., 1990).

In addition to $proANP_{1-126}$, a dimer of ANP_{99-126}, β-ANP, has been isolated and sequenced from extracts of human atria (Kangawa et al., 1984a,b, 1985; Oikawa et al., 1985). In vivo, β-ANP can be converted into ANP_{99-126}, and it has been shown to have a longer half-life and more prolonged activity than ANP_{99-126}. β-ANP is present in human plasma (Miyata et al., 1987), and its concentration has been shown to increase in atria and plasma during severe CHF and aging (Akimoto et al., 1988a; Marumo et al., 1988; Sugawara et al., 1988b,c). It is not yet clear whether the formation of this dimer represents a normal posttranslational mechanism or whether its abnormal increased level in chronic CHF has pathophysiological significance. Small quantities of prohormone have also been detected in plasma in humans and experimental animals with severe CHF (see below).

Several studies have indicated that the major form of NH_2-terminal ANP-related material is similar to $proANP_{1-98}$ in plasma, perfusate from the isolated rat heart preparation, and incubation medium of cultured myocytes (Katsube et al., 1986; Michener et al., 1986; Bloch et al., 1987a; Nakao et al., 1987; Glembotski et al., 1988; Itoh et al., 1988a; Murthy et al., 1988; Sundsfjord et al., 1988; Thibault et al., 1988). Primary atrial myocytes in the presence of dexamethasone also secrete NH_2-terminal ANP-related material that is chromatographically indistinguishable from $proANP_{1-98}$ prepared by thrombin cleavage of $proANP_{1-126}$ (Glembotski et al., 1988). The IR-NT-ANP is released by the heart in equimolar amounts with ANP_{99-126} and cosecreted with ANP_{99-126} in response to atrial stretch both in vitro (Dietz et al., 1991) and in vivo (Itoh et al., 1988a; Sundsfjord et al., 1988). In vivo, for example, vasopressin administration (Michener et al., 1986) and volume loading (Kukkonen et al., 1992) increase plasma IR-NT-ANP concentrations. Plasma levels of IR-NT-ANP in humans (Itoh et al., 1988a; Sundsfjord et al., 1988) and experimental animals are higher than those for ANP_{99-126}, indicating differences in the mechanisms of elimination between the two fragments. Essentially all of the plasma IR-NT-ANP is cleared by the kidney (Katsube et al., 1986; Itoh et al., 1988a; Sundsfjord et al., 1988), which filters and presumably degrades it on the brush border of the proximal tubules. The half-life of IR-NT-ANP in rats was found to be 8-fold longer than that of its COOH-terminal counterpart (2.5 min versus 20 s) (Thibault et al., 1988). Attempts to demonstrate biological activities of $proANP_{1-98}$ have failed, probably because of the absence of specific receptors for the NH_2-terminal fragment (Thibault et al., 1988). These observations support the concept that the COOH-terminal ANP and NH_2-terminal metabolites of $proANP_{1-126}$ are released together from atria and that measurements of NT-ANP can be used to characterize endogenous secretion of ANP.

Recent data have suggested that other smaller peptides derived from the NH_2-terminal region of human $proANP_{1-98}$, $proANP_{1-30}$, $proANP_{31-67}$, and $proANP_{79-98}$ may have cardiovascular functions (Vesely et al., 1987a,b; Winters et al., 1988). All three are reported to relax porcine aortas and stimulate the production of cGMP by aortic tissue (Vesely et al. 1987b). The three peptides produced identical dose-response curves for the stimulation of guanylate cyclase. $ProANP_{1-30}$ and $proANP_{31-67}$ were also reported to increase cGMP levels in whole kidney homogenates (Vesely et al., 1987a). In preliminary experiments, $proANP_{1-30}$ and $proANP_{31-67}$ were found to have different binding sites from ANP (Vesely et al., 1990). Furthermore, in contrast to ANP, $proANP_{1-30}$ and $proANP_{31-67}$ did not affect ANG II-stimulated aldosterone secretion in calf adrenal cells (Denker et al., 1990). In anesthetized rats, all three peptides decreased blood pressure, and $proANP_{31-67}$ had the most potent natriuretic effect (Martin et al., 1990). Recently, $proANP_{31-67}$ was shown to inhibit sodium reabsorption in inner medullary collecting duct cells equal to ANP at physiological concentrations, but their mechanisms of action appeared to be different (Gunning et al., 1992). $ProANP_{31-67}$, in contrast to ANP, appears to inhibit the Na^+/K^+-ATPase in the inner medullary collecting duct, an effect mediated by the generation of PGE_2 (Gunning et al., 1992). Immunocytochemical studies localized $proANP_{1-30}$ and $proANP_{31-67}$ to the pars convoluta and pars recta of the proximal convoluted tubules in a sub-brush border location and to a lesser extent also in the distal tubule (Ramirez et al., 1992).

Radioimmunoassays using antibodies raised to $proANP_{1-30}$ and $proANP_{31-67}$ reacted with human plasma (Winters et al., 1988) and detected markedly enhanced immunoreactivity in the plasma of patients with chronic renal failure (Winters et al., 1988). Evaluation of human plasma that had undergone reverse-phase high-performance liquid chromatography suggested that $proANP_{1-30}$ and $proANP_{31-67}$ were distinct peaks in human plasma corresponding to the peaks of the pure synthetic peptides (Winters et al., 1989). Molecular weight determination revealed that the $proANP_{1-30}$ radioimmunoassay recognized a peptide similar to the complete NH_2-terminal fragment, $proANP_{1-98}$, whereas the $proANP_{31-67}$ radioimmunoassay detected a peptide with a molecular weight of 3900 to 4000, consistent with amino acids 31 to 67 of the prohormone (Winters et al., 1989), suggesting that the midportion of the NH_2 terminus of proANP also circulates in plasma. Furthermore, $proANP_{31-67}$ immunoreactivity has been reported to be secreted simulta-

neously with $proANP_{1-98}$ and ANP_{99-126} from isolated rat atria in response to atrial distension (Dietz et al., 1991). Unfortunately, the exact amino acid sequence or the number of amino acids in the latter peptide from the plasma or perfusate have not yet been determined. Furthermore, studies in which several antibodies directed against the NH_2 terminus of proANP (amino acids 1 to 16, 1 to 25, 1 to 30, 11 to 37, 31 to 67, 48 to 67, 87 to 98) were used with chromatographic separation failed to detect significant amounts of low molecular weight peptides in plasma but detected a single high molecular weight peptide, presumably representing the full-length $proANP_{1-98}$ (Gerbes and Vollmar, 1988; Itoh et al., 1988a; Meleagros et al., 1988; Sundsfjord et al., 1988; Thibault et al., 1988; Buckley et al., 1989; Meleagros et al., 1989; Buckley et al., 1990a,b; Chen et al., 1990a; Vollmar, 1991). Finally, primary cell cultures secreted only ANP_{99-126} and $proANP_{1-98}$, suggesting that the formation of other truncated peptides derived from either the NH_2 or COOH terminus of ANP could be the result of proteolytic cleavage of proANP (Glembotski et al., 1988). Thus, until the $proANP_{31-67}$ immunoreactive material is definitively characterized in human plasma, the physiological role of peptides from the NH_2 terminus of proANP in the regulation of blood volume and pressure remains unclear.

2. Site of atrial natriuretic peptide processing and processing enzymes. Whether the conversion of $proANP_{1-126}$ to ANP_{99-126} occurs within the secretory granule, during or after release, has not yet been clarified. In some studies, proteases from serum (Bloch et al., 1985, 1987a,b; Glembotski and Gibson, 1985), platelets (Trippodo et al., 1985), or plasma (Itoh et al., 1987a) were reported to convert $proANP_{1-126}$ to ANP_{99-126}. This suggests that ANP_{99-126} might be generated from $proANP_{1-126}$ in the circulation. However, ANP_{99-126} is released from isolated perfused hearts (Currie et al., 1984b; Lang et al., 1985; Michener et al., 1986; Ruskoaho et al., 1986a; Saito et al., 1986; Thibault et al., 1986; Nakao et al., 1987; Shields and Glembotski, 1987; Toki et al., 1990) and isolated atria (Vuolteenaho et al., 1985b) in the absence of blood components. $ProANP_{1-126}$ has not been detected in blood collected directly from the coronary sinus of the heart (Yandle et al., 1987), and plasma is an inefficient catalyst of proANP processing (Michener et al., 1986; Gibson et al., 1987; Murthy et al., 1988), further showing that the posttranslational cleavage to form ANP_{99-126} does not require proteases from the circulation. Therefore, the processing of ANP occurs within or close to the cardiac myocyte itself and, because there is little ANP_{99-126} stored within the myocyte, processing must occur almost simultaneously with secretion.

$ProANP_{1-126}$ is mainly released into the culture medium in primary cultures of neonatal or adult rat cardiocytes cultured in serum-free medium (Bloch et al., 1985, 1986, 1987a,b; Glembotski and Gibson, 1985; Sylvestre et al., 1986; Cantin et al., 1987; Glembotski et al., 1987; Zisfein et al., 1987; Gardner et al., 1988; Hassall et al., 1988). Neonatal cardiocytes cultured in the presence of hydrocortisone and dexamethasone cleave $proANP_{1-126}$ to release ANP_{99-126} (Shields and Glembotski, 1988; Shields et al., 1988). Dexamethasone stimulates ANP processing and secretion in a dose-dependent, reversible manner in both atrial and ventricular cells (Shields et al., 1988) and is also stimulated by the specific glucocorticoid receptor agonist, RU 28362. Both dexamethasone- and RU 28362-stimulated ANP processing are inhibited by the specific glucocorticoid receptor antagonist, RU 38486. Interestingly, neonatal ventricular myocytes, which possess few granules, can also process ANP in a glucocorticoid-dependent manner (Shields et al., 1988). Appropriate processing of proANP by cardiocytes supplemented with steroids further suggests that prohormone cleavage is a myocyte function and that the enzyme responsible for this is induced by dexamethasone treatment. Thus, the posttranslational processing of $proANP_{1-126}$ to ANP_{99-126} probably occurs within, or close to, the cardiac myocytes.

The final posttranslational processing does not have to take place within or on the surface of the myocyte: it could occur in another cell such as endothelial cells or fibroblasts. Page et al. (1986) suggested the involvement of the endoplasmic reticulum and Golgi apparatus in the processing of proANP just before secretion as active ANP. A membrane-bound serine protease (named atrioactivase) cleaving proANP between positions 98 and 99 has been reported (Imada et al., 1987, 1988; for review, see Inagami, 1989), and this finding, together with the fact that proANP repeatedly perfused through isolated hearts is partially converted to an ANP_{99-126}-like peptide (Michener et al., 1986; Toki et al., 1990), supports the suggestion that a membrane-bound ANP-processing enzyme, a cell surface protease, may be involved in the conversion of $proANP_{1-126}$ to ANP_{99-126}. Furthermore, proANP processing in isolated perfused rat hearts is inhibited by high concentrations of aprotinin (Ito et al., 1988; Toki et al., 1990). There are invaginations, termed caveolae, on the surface of atrial myocytes that seem to be areas where granule fusion with the plasma membrane occurs (Page et al., 1986). If these caveolae contain a processing enzyme, proANP could be processed either on the cell surface or as it diffuses into the extracellular milieu. In agreement, the results of Corthorn et al. (1991) suggest that rat atrial granules may not contain active processing enzyme.

However, results of other studies suggest that (*a*) exogenous $proANP_{1-126}$ is not cleaved by the isolated perfused heart (Currie et al., 1984b; Gibson et al., 1987), (*b*) aprotinin in the cell culture system inhibited ANP processing only at unusually high concentrations (Glembotski et al., 1991), and (*c*) there was no apparent conversion of labeled $proANP_{1-126}$ by medium from actively processing atrial cell cultures (Shields et al., 1988; Sei et

al., 1992). The observation that no significant decrease in proANP$_{1-126}$ processing occurs in cytosine arabinoside-treated cell cultures when compared with control cultures (Sei et al., 1992) also suggests that maturation of proANP occurs simultaneously with secretion, probably within the myocyte. Furthermore, coupling biosynthetic labeling with timed secretion experiments showed that the extent of ANP processing in primary atrial cultures was not dependent on the time after secretion (Sei et al., 1992); maximal levels of processing were observed at all secretion times examined, suggesting that the cleavage event takes place cosecretionally.

Granules predominantly contain proANP$_{1-126}$, and, if the processing enzyme is located in atrial granules, there must be a mechanism preventing its cleavage until the appropriate stimuli for hormone release occurs. Processing could occur intracellularly if an ANP-processing enzyme is present within secretory granules in an inactive form or if proANP$_{1-126}$ and active ANP-processing enzymes are contained in separate granules within the myocyte. Three findings support the presence of an inactive processing enzyme within the granules: (*a*) the relatively high [Ca^{2+}] of atrial secretory granules (Somlyo et al., 1988); (*b*) the possible binding of Ca^{2+} by the acidic part of proANP (Thibault and Doubell, 1992) or chromogranins (Steiner et al., 1990); and (*c*) a putative, Ca^{2+}-dependent, proANP-processing enzyme that has been isolated from the inner membrane of atrial secretory granules (Wypij and Harris, 1988, 1992). At high [Ca^{2+}] and ionic strength, this serine proteinase would be completely inhibited (Harris, 1989). The enzyme preferentially hydrolyzes the Arg^{98}-Ser^{99} bond which, after prolonged incubation, then serves as the substrate for the formation of ANP$_{103-126}$. Thus, in response to the appropriate stimulus for release, intragranular conditions (Ca^{2+}, ionic strength, pH, substrate dilution, or other mechanisms) may be altered to increase this or other putative processing enzymes' activity, producing ANP$_{99-126}$ immediately prior to or during the release of granular contents.

Several other proteolytic enzymes have been suggested to process proANP$_{1-126}$ to ANP$_{99-126}$ in addition to the two serine proteases, carboxypeptidase E (H) and the blood proteases already described. These include kallikrein, trypsin, and thrombin (Currie et al., 1984a; Thibault et al., 1984; Michener et al., 1986; Gibson et al., 1987) which appear in vitro to cleave high molecular weight proANP-like peptides to low molecular weight peptides. Atrial zinc-metallodipeptidyl carboxyhydrolase, present in the microsomes and atrial granules (Harris and Wilson, 1984, 1985a), converts ANP$_{103-125}$ to ANP$_{103-123}$ (Harris and Wilson, 1985b) and can remove the tripeptide, Phe-Arg-Tyr, from the COOH terminus of ANP$_{103-126}$ to form ANP$_{103-123}$ (Harris, 1989). The thiol proteinase identified in a detergent homogenate prepared from whole atrial tissue (Baxter et al., 1986) is probably lysosomal cathepsin B and is unlikely to be involved in the physiological processing of proANP$_{1-126}$ (Wypij and Harris, 1988). Seidah et al. (1986) purified an IRCM serine protease from heart atrium and ventricle that yields the ANP$_{103-126}$ > ANP$_{102-126}$ > ANP $_{99-126}$ (Seidah et al., 1986).

Atrial granules also contain peptidylglycine-amidating monooxygenase, but its function is unclear because ANP is not an α-amidated peptide (Harris, 1989). Cathepsin B, a lysosomal protease, has recently been localized in rat right atrial secretory granules (Watanabe et al., 1989) and could also play a role in catalyzing the processing reaction. Finally, arginine esterase A, a protease of the kallikrein gene family, with an alkaline pH optimum, was found with the prohormone in granules and was capable of cleaving the proANP to a low molecular weight product (Simson et al., 1989). Whether one or several of these enzymes are physiologically important for prohormone cleavage remains an area of active research.

The ANP gene is also expressed on several extracardiac cells. It seems that the paired basic amino acids at positions 101 and 102 (-Arg^{98}-Ser^{99}-Leu^{100}-Arg^{101}-Arg^{102}-Ser^{103}-) may serve as a maturation site for processing in some noncardiac cell types showing the presence of alternative models of ANP prohormone processing. Two major forms of ANP isolated from the hypothalamus, pituitary, and other regions of the brain, containing 25 and 24 amino acids and representing cleavage products of proANP$_{1-126}$ at the Arg_{101}-Arg_{102} (ANP$_{102-126}$) and Arg_{102}-Ser_{103} (ANP$_{103-126}$) bonds, have been found (Glembotski et al., 1985; Shiono et al. 1986; Ueda et al. 1987; Shields et al., 1990). Significant differences in the structure of the bioactive ANP between atria and brain most likely result from posttranscriptional events in the individual tissues. Processing of proANP$_{1-126}$ in the brain may be catalyzed by proteases specific for double basic residues, i.e., sequences that are commonly found in the processing of precursors of many peptide hormones (Harris, 1989). A likely candidate processing enzyme for neural proANP$_{1-126}$ is IRCM serine protease which has the requisite substrate specificity and cellular localization (Seidah et al., 1986).

In summary, some aspects of the processing of ANP are unusual when compared with other peptide hormone systems, and the activation of proANP$_{1-126}$ is thus an ideal model system for the study of prohormone conversion. ANP prohormone processing does not appear to occur during storage because proANP$_{1-126}$, rather than mature ANP$_{99-126}$, is the main storage form in the secretory vesicles. The activation of proANP$_{1-126}$ probably either occurs intracellularly or simultaneously during release from the atrial myocyte when an extracellular stimulus initiates secretion, possibly by simultaneously changing the activity of the processing enzyme(s). Several enzymes have been suggested to be responsible for the proteolytic processing of proANP$_{1-126}$, and some of

them have been shown to be selective for the Arg^{98}-Ser^{99} bond and are found in atrial granules or in the cellular membrane. Whether one of these enzymes is physiologically important for ANP prohormone cleavage is not known. As yet, we still do not know whether a unique, hitherto undescribed, enzyme exists for ANP processing or whether one of the enzymes described above has a physiological role in the processing of $proANP_{1-126}$ into circulating NH_2-terminal $proANP_{1-98}$ and COOH-terminal ANP_{99-126}. The details of the mechanism regulating the processing of proANP have still to be clarified, and more information is needed concerning the properties and function of the proteolytic enzymes to permit the development of drugs to influence the ANP hormonal system.

D. Extracardiac Expression of Atrial Natriuretic Peptide

ANP-specific mRNA or proANP-like immunoreactivity has been described in aortic arch cells (Gardner et al., 1987a), lung (Gardner et al., 1986a, 1987c), brain (Jacobowitz et al., 1985; Saper et al., 1985; Gardner et al., 1987c; Standaert et al., 1988), adrenals (Ong et al., 1987; Morel et al., 1988), kidney (Sakamoto et al., 1985; McKenzie at al. 1985; Flugge et al., 1987; Ritter et al., 1991), gastrointestinal tract (Vollmar et al., 1988; Vuolteenaho et al., 1988), thymus (Vollmar and Schulz, 1988; Vollmar et al., 1990), and chorioidea and ciliary bodies (Gaspar et al., 1991). IR-ANP levels in these tissues are, however, far lower than those in atriocytes, and they are, therefore, unlikely to contribute substantially to plasma levels, at least under physiological conditions (for reviews, see Gutkowska and Nemer, 1989; Vollmar, 1990). The ANP transcripts in all of the extracardiac sites are identical with but <1 to 2% of those seen in the cardiac atria (Gardner et al., 1986a, 1987a,c).

ANP is synthesized in several discrete loci within the central nervous system and may thus act as a neurotransmitter or neuropeptide to regulate blood pressure and fluid and electrolyte balance. ANP-positive neurons have been identified in several well-defined regions of the brain by immunohistochemistry and radioimmunoassay (Tanaka et al., 1984; Jacobowitz et al., 1985; Kawata et al., 1985; Saper et al., 1985; Skofitsch et al., 1985; Standaert et al., 1986; for reviews, see Genest and Cantin, 1988; Skofitsch and Jacobowitz, 1988; Gutkowska and Nemer, 1989; Inagami et al., 1989; Saavedra, 1989). These neurons are especially dense within the hypothalamus (paraventricular nuclei that synthesize vasopressin and influence anterior pituitary and autonomic nervous system function) and the AV3V region, which is believed to be important in blood pressure regulation. In the central nervous system, ANP gene transcripts have been especially found in the anterior pituitary (Gardner et al., 1986a, 1987c), hypothalamus (Gardner et al., 1986a, 1987c), pontine brainstem, and several limbic structures (Gardner et al., 1987c). Detectable, but very low, levels of ANP mRNA (<0.1% of the atrial transcript level) are also present in the cerebral cortex and in the cerebellum (Gardner et al., 1987c; Standaert et al., 1988). Specific ANP mRNA has been demonstrated by the polymerase chain reaction technique in ciliary body and chorioidea tissue extracts but not in the retina of the rat eye (Gaspar et al., 1991).

In vitro, IR-ANP is released from the hypothalamus in a Ca^{2+}-dependent manner in response to K^+ depolarization (Shibasaki et al., 1986; Tanaka and Inagami, 1986a), suggesting that ANP may play the role of neuromodulator. The change in [Na^+] (Shibasaki et al., 1988), β-adrenergic agonists (Bush et al., 1990), and forskolin (Huang et al., 1991) also stimulate IR-ANP secretion from rat hypothalamic tissues in vitro, whereas dexamethasone and triiodothyronine suppress both the release of IR-ANP and the levels of ANP mRNA in the neuron-enriched primary cultures from fetal rat brains (Deschepper et al., 1990). Furthermore, norepinephrine and epinephrine, acting through α_2-adrenoceptors, facilitate IR-ANP secretion and ANP expression in long-term cultures of rat hypothalamic neurons (Huang et al., 1992). ANP is released into hypophyseal portal blood where its concentration is three to four times greater than in peripheral plasma (Lim et al., 1990). Ovariectomy and estradiol treatment decreased the portal blood IR-ANP concentrations (Sheward et al., 1991), further supporting the role of ANP role in the modulation of pituitary hormone release.

Brain ANP appears to be regulated by cardiovascular homeostasis and systemic blood pressure, although the regulation of hypothalamic peptide levels has been shown to be discordant with that of its atrial equivalent (Tanaka et al., 1984; Morii et al., 1986b; Ruskoaho and Leppäluoto, 1988a). Thus, tissue-specific factors appear to be operative in modulating the magnitude and direction of ANP regulation. Changes in IR-ANP have been found in discrete brain regions after dehydration (Samson, 1985), dietary salt load and restriction (Tanaka et al., 1984; Saavedra, 1989; John and Morich, 1990; Palkovits et al., 1990; Jin et al., 1991), adrenalectomy (Palkovits et al., 1987; Geiger et al., 1991), or aldosterone and dexamethasone treatment (Geiger et al., 1991). Acute volume expansion can also induce significant increments in IR-ANP concentration of cerebral spinal fluid drawn from cisterna magna (Cameron and Espiner, 1990).

Several lines of evidence also show that brain ANP is altered in hypertensive rats compared with control rats. Studies from a number of laboratories have demonstrated that the IR-ANP content of the hypothalamus and some other brain areas is significantly elevated in hypertensive rats compared with normotensive controls (Imada et al., 1985; Morii et al., 1986a; Takayanagi et al., 1986; Jin et al., 1988a; Ruskoaho and Leppäluoto, 1988a; Debinski et al., 1989; Geiger et al., 1989; Bahner et al., 1991; Komatsu et al., 1992), whereas other groups

have reported that IR-ANP levels in specific brain nuclei of SHR are lower than in WKY rats (Bahner et al., 1988; Jin et al., 1991). In a recent study, in which quantitative polymerase chain reaction was used, Chen et al. (1992) demonstrated the presence of ANP mRNA in the hypothalamus of SHR. They found regional nonhomogeneity of ANP mRNA in the hypothalamus of SHR and WKY rats but did not detect significant differences in ANP mRNA levels in hypothalamic areas between SHR and WKY rats (Chen et al., 1992). Yet, an RNase protection assay revealed that the concentrations of ANP mRNA in the hypothalamus and brainstem of 17-week-old SHR were higher than those of age-matched WKY rats (Komatsu et al., 1992), suggesting that elevated levels of ANP in the central nervous system result from increased synthesis of ANP. Two other recent studies have provided more evidence that endogenous ANP in brain mediates blood pressure control in the SHR. Anterior hypothalamic microinjection of monoclonal antibody to ANP caused significant dose-related decreases in mean arterial pressure and heart rate in SHR but not WKY rats (Yang et al., 1990). Furthermore, microinjection of monoclonal antibody (the same used in the previous study) into the caudal nucleus tractus solitarii caused significant increases in mean arterial pressure in SHR but not WKY rats (Yang et al., 1992), suggesting that the endogenous ANP in this area mediates tonic control of blood pressure in SHR. Taken together, these data show a significant role for this peptide in the central regulation of blood pressure and fluid and electrolyte homeostasis.

Peripheral neural tissues have been found to be additional sites of ANP storage. Rat adrenal chromaffin cells have been shown by immunohistochemical staining to contain ANP in epinephrine-containing cells and, to a lesser extent, in norepinephrine-containing cells (McKenzie et al., 1985; Inagaki et al., 1986; Ong et al., 1987; Morel et al., 1988; Nguyen et al., 1990). Sequence analysis of purified ANP immunoreactivity from adrenal medullary extracts has identified both ANP_{99-126} and proANP (Ong et al., 1987). The presence of ANP immunoreactivity in homogenates from human pheochromocytoma was reported by Chen et al. (1990b). Morel et al. (1988), using an in situ hybridization method, showed the presence of ANP mRNA in about 15% of rat adrenal medulla, suggesting that ANP is synthesized in the adrenal gland. Furthermore, cultured bovine adrenal chromaffin cells can be stimulated to produce and secrete ANP (Gutkowska and Nemer, 1989; Nguyen et al., 1990), and the stimulation of splanchnic nerve may specifically enhance the uptake of ANP by the adrenal gland (Edwards et al., 1990a). ANP has been detected also in cell bodies of both the cholinergic and adrenergic ganglions in rats. ANP-positive cells were first identified by immunohistochemical staining in peripheral ganglia in the rat superior cervical ganglion (Papka et al., 1985). Subsequently, ANP was detected in extracts of both the ganglion nodosa, superior cervical ganglion, and sparsely distributed SIF cell bodies of the superior celiac mesenteric ganglion and primary sensory neurons (Debinski et al., 1986, 1987; Morii et al., 1987; Kuchel et al., 1988; Nohr et al., 1989; for reviews, see Gutkowska and Nemer, 1989; Debinski et al., 1990). In addition, ANP-like immunoreactivity is present in the chemosensitive cells of the cat carotid body (Wang et al., 1991), and ANP mRNA levels are approximately 5- to 10-fold higher in the aortic arch, the site of the vascular baroreceptors, than in the distal aortic tissue (Gardner et al., 1987a). The peptide in the arch is largely confined to the adventitia and is especially dense around the bifurcation of the major cephalic vessels.

The development of the vena cavae and the pulmonary veins in embryos is closely associated with the development of the atria. This explains the presence of cardiac muscle cells in the wall of the great thoracic and pulmonary veins as well as the presence of specific granules in these muscle cells. Strong ANP immunoreactivity (Asai et al., 1987; Springall et al., 1988; Toshimori et al., 1988a) as well as ANP mRNA (Springall et al., 1988; Sola et al., 1990) has been shown by in situ hybridization in both extrapulmonary and intrapulmonary veins and vena cavae of the rat. The inferior vena cava contains almost as much ANP mRNA as the atria, whereas the levels are lower in the superior vena cava but higher than in normal ventricles (Sola et al., 1990). The presence of proANP in these veins further supports the suggestion of local ANP synthesis (Springall et al., 1988; Sola et al., 1990). ANP immunoreactivity appears particularly intense in myocytes in the junctional area between the right atrium and both the inferior and superior vena cavae and decreases further away from the heart (Larsen et al., 1987; Larsen, 1988). These extracardiac sites may be an additional source of circulating ANP.

The concentration of ANP mRNA in the lungs is several hundred-fold less than that in atria (Gardner et al., 1986a). Immunocytochemistry has demonstrated ANP-positive ovoid cells in the peripheral alveolar walls of the lung (Gardner et al., 1986a). ANP prohormone has been identified in lung extracts (Asai et al., 1987), but lung tissue also contains low molecular weight ANP that is thought to accumulate by extraction of ANP_{99-126} from the plasma. The fact that ANP mRNA has been detected by in situ hybridization in extra- and intrapulmonary veins suggests that the ANP mRNA and proANP detected in the lungs may originate in these veins (Springall et al., 1988). Primary cultures of lung tissue and isolated perfused rat lungs, from which ANP is released as a 28-amino acid peptide, show that ANP is secreted from the lung (Gutkowska et al., 1987, 1989; Gutkowska and Nemer, 1989). Furthermore, a variety of factors regulate ANP expression in the lung. Thus, water deprivation decreases the amount of IR-ANP (Sakamoto et

al., 1986), high salt intake increases lung IR-ANP concentrations (Widimsky et al., 1990a), and increased pulmonary levels of IR-ANP and ANP mRNA have been found in cardiomyopathic hamsters (Currie et al., 1987). Glucocorticoids and thyroid hormones also stimulate ANP gene expression in primary cultures of pneumocytes (Matsubara et al., 1988b), and ectopic ANP expression has been identified in small cell lung cancer (Bliss et al., 1990). The physiological importance of ANP in lungs remains to be clarified, but pulmonary ANP could have local relaxant effects on either pulmonary vascular or bronchial smooth muscle and may, perhaps, contribute to circulating ANP levels.

A number of other tissues have been shown to contain IR-ANP (for reviews, see Forssmann et al., 1989; Gutkowska and Nemer, 1989; Vollmar, 1990). Low molecular mass IR-ANP, together with ANP histochemical immunoreactivity in kidney extracts (Sakamoto et al., 1985) and kidney (McKenzie et al., 1985) were early findings. Water deprivation for 5 days and perfusion with saline decreased IR-ANP in the intact kidney (Sakamoto et al., 1985). Recent data have further demonstrated that the ANP-like prohormone is synthesized and constitutively secreted together with low molecular weight peptide by primary cultures of neonatal and adult renal cells (Greenwald et al., 1991; Ritter et al., 1991; see section II.E.1). The gastrointestinal tract is also a target organ for ANP, and ANP appears to be a member of the peptides present in both the brain and intestine. ANP immunoreactivity was found in the rat stomach and small intestine (Vuolteenaho et al., 1988) and in the pig jejunum and colon (Vollmar et al., 1988). In support of endogenous production of ANP, high-performance liquid chromatography analysis revealed the high molecular weight peptide precursor in tissue extracts (Vollmar et al., 1988; Vuolteenaho et al., 1988). ANP was further shown to stimulate gastric acid secretion after i.c.v. administration in anesthetized rats (Puurunen and Ruskoaho, 1987). Vollmar et al. (1988) also demonstrated the presence of ANP precursor material in the lymphoid tissue of the intestine. A further link of ANP with the immune system is shown by the existence of mRNA for ANP in the rat thymus gland (Vollmar and Schulz, 1988; Vollmar et al., 1990). Other tissues shown to contain IR-ANP include corpus luteum, human skin, pancreas, thyroid gland, spleen, testis, liver, salivary glands, salt gland of the Peking duck, and lacrimal gland of the domestic pig (Cantin et al., 1984; Gutkowska and Nemer, 1989; Lange et al., 1989, 1990; Vollmar, 1990; Sellitti and Hughes, 1990; Hughes and Sellitti, 1991; Pandey and Orgebin-Christ, 1991; Vollmar et al., 1991; Vesely et al., 1992). At this point, the importance of ANP in these tissues is still unclear.

E. Other Atrial Natriuretic Peptide-like Peptides

Several other compounds that are closely related to ANP have been independently identified in several laboratories. Isolation of these new peptides has been facilitated by both molecular and biochemical features that are shared by members of the natriuretic peptide hormone family as well as by assays for new peptides with muscle relaxant activity. The peptides are classified into A, B, or C types based on their genetic or amino acid homology with the prototypes; ANP (A type), BNP (B type), or CNP (Sudoh et al., 1990; Rosenzweig and Seidman, 1991). There is a significant amount of structural and amino acid sequence homology among the A, B, and C types, but they do not all produce exactly similar biological effects. Furthermore, in the 3′-untranslated regions of the cDNAs encoding ANP, BNP, and CNP precursors, a characteristic nucleotide sequence (ATTTA), which changes turnover rates of mRNA, is found at a different frequency in each cDNA (Seilhamer et al., 1989; Kojima et al., 1990; Akizuki et al., 1991). In addition, CNP interacts with a B-type bioactive receptor, whereas ANP and BNP bind to an A-type receptor (Koller et al., 1991); CNP is principally localized in the central nervous system, whereas ANP and BNP are mainly present in the heart. Thus, although the three natriuretic peptides, ANP, BNP and CNP, have a highly homologous structure, the differences in the receptors, cDNA structure, and tissue distribution may reflect their functional differences in the regulation of volume and pressure homeostasis. Finally, a recently isolated 36-amino acid peptide from eel cardiac ventricles (termed VNP) may represent an additional type of natriuretic peptide. The primary structure of eel VNP is characterized by its long COOH-terminal tail sequence (Takei et al., 1991) not found in mammals. Endogenous molecular forms and processing pathways of the three types of human natriuretic peptides are summarized in figure 3.

1. Urodilatin. Early studies demonstrated the existence of a low molecular mass ANP immunoreactivity in kidney extracts (Sakamoto et al., 1985) and urine (Marumo et al., 1986) as well as ANP immunoreactivity by immunohistochemistry in the kidney (McKenzie et al., 1985). Because there is a high ANP receptor-binding capacity in the kidney, it was presumed that this renal ANP was of blood origin. However, in 1988, a new peptide named urodilatin was identified in human urine using a smooth muscle dilation bioassay (Schulz-Knappe et al., 1988; for reviews, see Goetz, 1991; Kentsch et al., 1991). The amino acid analysis showed that urodilatin is a 32-amino acid peptide identical in sequence with ANP_{99-126} but with an NH_2-terminal extension of four amino acid residues (Thr-Ala-Pro-Arg), which can also be found in the $proANP_{1-126}$. Urodilatin (denoted as ANP_{95-126} in this review) cannot be detected in plasma (Saxenhofer et al., 1990), suggesting that it is produced and processed in the kidney. ANP_{95-126} appears not to be a substrate for endopeptidase in renal cortical membrane preparations (Gagelmann et al., 1988) and seems to be excreted unaltered in the urine (Solc et al., 1991). Recent data have

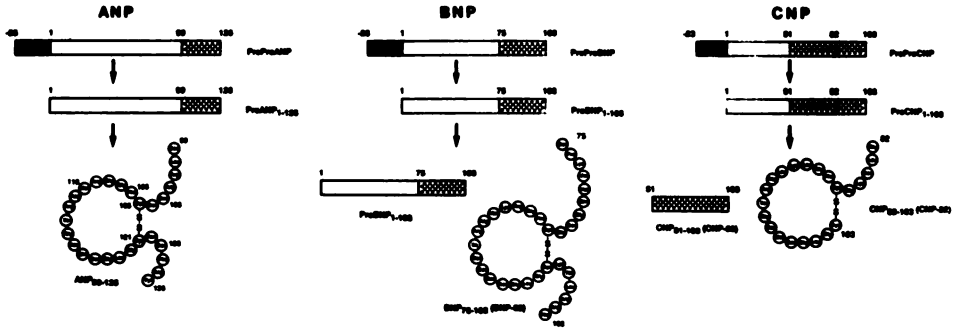

FIG. 3. Proteolytic processing pathways and major molecular forms of the three types of natriuretic peptides in human circulation and central nervous system (modified from Minamino et al., 1991).

further demonstrated that ANP-like prohormone is synthesized and constitutively secreted by primary cultures of neonatal and adult renal cells (Ritter et al., 1991). The site of synthesis has been localized to distal cortical nephrons, and proANP synthesis is up-regulated in a model of experimental nephrosis in the rat (Greenwald et al., 1991). These results suggest that ANP_{95-126} is probably generated by tubular epithelial cells within the renal cortex as a different posttranslational processing product of $proANP_{1-126}$, presumably secreted into the tubular lumen, and induces natriuresis and diuresis by acting on the inner medullary collecting duct ANP receptors.

The biological effects of ANP_{95-126} resemble the actions of ANP_{99-126} (Goetz, 1991; Kentsch et al., 1991). In the bovine adrenal cortex, ANP_{95-126} was shown to activate particulate guanylate cyclase and generate cGMP (Heim et al., 1989, 1992). In the collecting ducts of the rat kidney, ANP_{95-126} inhibits sodium and water reabsorption (Schulz-Knappe et al., 1990). In a placebo-controlled, randomized crossover study, ANP_{95-126} acutely modified renal and cardiovascular function in healthy men (Saxenhofer et al., 1990). Injection i.v. of ANP_{95-126} in normal subjects dose dependently enhanced GFR and sodium, water, and cGMP excretion, and in higher doses it also decreased diastolic blood pressure. In healthy volunteers, the most striking effect of ANP_{95-126}, compared with ANP_{99-126}, was marked and long-lasting reduction of pulmonary arterial and pulmonary capillary wedge pressures (Kentsch et al., 1992). In normal dogs and dogs with heart failure, incremental infusions of the ANP_{95-126} exerted similar hemodynamic effects to those of ANP_{99-126} (Riegger et al., 1990; Villarreal et al., 1991). In contrast to the considerable attenuation of the renal effect of ANP_{99-126} in heart failure, ANP_{95-126} produced a significant diuresis and natriuresis in dogs both before and after the induction of heart failure (Riegger et al., 1990; Villarreal et al., 1991). Interestingly, sodium excretion correlated better with urine ANP_{95-126} excretion than it did with circulating plasma IR-ANP concentration in dogs either during spontaneous sodium excretion or in response to saline infusion and left atrial distension (Goetz et al., 1990), as well as in humans in response to saline infusion (Kentsch et al., 1991). In pentobarbital sodium-anesthetized dogs, ANP_{95-126} appeared to be a more effective natriuretic and diuretic peptide than ANP_{99-126} (Hildebrandt et al., 1992). These findings suggest that ANP has a paracrine function in the kidney. However, the hypothesis that ANP_{95-126} or urodilatin, rather than blood-derived ANP, could regulate natriuresis and diuresis under normal physiological conditions remains to be established.

2. Brain natriuretic peptide and related peptides. Other compounds, BNP and CNP that have recently been isolated, are closely related but clearly distinct from ANP, indicating that both BNP and CNP are different gene products from that of ANP. BNP was first identified in porcine brain as either a 26-amino acid peptide (Sudoh et al., 1988a) or its NH_2-terminally extended form (BNP-32) (Sudoh et al., 1988b). Like ANP, BNP contains a 17-member ring that is formed by a disulfide bond (Sudoh et al., 1988a). Within this ring structure, the amino acid sequence of porcine BNP differs from that of human ANP at only four residues. Greater divergence from ANP sequences is exhibited by the residues in the amino and COOH extensions of the BNP molecules (for structures, see Aburaya et al., 1991). Although originally isolated from the brain, BNP is more abundant in cardiac atria and ventricles than in the central nervous system (Minamino et al., 1988a,b; Dagnino et al., 1991). BNP is also secreted into the circulation from the porcine heart (Saito et al., 1989b), suggesting that BNP mainly functions as a circulating hormone rather than as a neuropeptide in the central nervous system.

Like ANP, BNPs are derived from a common precursor, termed preproBNP, which contains between 121 and 134 amino acids depending upon species. Cloning and sequence analysis of cDNAs for porcine (Maekawa et al., 1988; Porter et al., 1989a), rat (Kojima et al., 1989), dog (Seilhamer et al., 1989), and human (Seihamer et al., 1989; Sudoh et al., 1989) BNP precursors have shown that structural species differences observed among mammalian BNPs are much larger than those among mammalian ANPs, even in the COOH-terminal amino acid sequences essential for the biological activity. In the porcine heart, the removal of a 25-residue signal peptide

from a 131-residue preproBNP precursor leads to formation of the storage form, $proBNP_{1-106}$, from which circulating peptides BNP-26, BNP-29, and BNP-32 are processed (Minamino et al., 1988a,b; Maekawa et al., 1988; Aburaya et al., 1989a,b; Porter et al., 1989a; Saito et al., 1989b; Aburaya et al., 1991). In humans, BNP has been isolated from the atrial myocytes and brain and is a 32-amino acid peptide (Hino et al., 1990; Kambayashi et al., 1990; Minamino et al., 1991), which corresponds to the COOH-terminal sequence (77 to 108) of the human BNP precursor deduced from the cDNA sequence (Seilhamer et al., 1989; Sudoh et al., 1989). Rat BNP is mainly present as BNP-45 in the cardiac atrial myocytes and circulation (Aburaya et al., 1989a,b; Itoh et al., 1989a), and it is identical with the COOH-terminal sequence (51 to 95) of the rat $proBNP_{1-95}$ deduced from the cDNA sequence analysis (Kojima et al., 1989). Flynn et al. (1989) independently reported the sequence of iso-ANP, which consists of 45 amino acids isolated and sequenced from rat atriocytes and was initially believed to be sufficiently different from that of porcine BNP to suggest that iso-ANP represented a new hormone. However, subsequent nucleic acid sequence analyses of rat cDNA and genomic clones have demonstrated the relationship of this hormone to rat BNP-45 (Aburaya et al., 1989a,b; Kambayashi et al., 1989b; Kojima et al., 1989; Roy and Flynn, 1990; Pang et al., 1991). These data thus show that the proteolytic processing system of mammalian BNP is different in each species and that BNP-45 is the circulating form in rats and either BNP-32 or BNP-26 is found in the circulation in humans and pigs.

BNP has peripheral and central actions similar to those of ANP in experimental animals, which include a natriuretic, diuretic, and hypotensive effect and relaxation of smooth muscle (Sudoh et al., 1988a,b; Jennings and Flynn, 1990; Jennings et al., 1990). Like ANP, it induces a similar dose-related generation of cGMP in various cultured tissues. In normal humans, BNP has a similar range of effects (McGregor et al., 1990), but its clearance from the circulation is slower than that of human ANP (Mukoyama et al., 1991). In patients with chronic heart failure, BNP infusion improves left ventricular function by inducing vasodilation and a marked natriuresis (Yoshimura et al., 1991). The normal plasma level of IR-BNP in humans (0.9 to 1.5 pmol/liter) is about 4- to 6-fold lower than that of ANP (Mukoyama et al., 1990a,b, 1991). Chronic salt loading in man causes a similar increase in both plasma IR-BNP and IR-ANP levels (Lang et al., 1991a), although in patients with CHF and acute myocardial infarction (Mukoyama et al., 1991; Yoshimura et al., 1991), plasma IR-BNP levels increase much more markedly than do IR-ANP levels. In patients with myocardial infarction, the plasma IR-BNP concentration correlated inversely with the cardiac index (Mukoyama et al., 1991), suggesting that BNP may serve as a useful marker of left ventricular dysfunction. Furthermore, high plasma IR-BNP levels were observed during supraventricular tachycardia when compared with normal levels (Kohno et al., 1992a), and plasma IR-BNP concentration is elevated in patients with chronic renal failure and decreases during hemodialysis (Lang et al., 1992a). These findings show the significance of BNP as a new cardiac hormone and suggest that, like ANP, BNP regulates blood pressure and volume.

Aldosterone secretion inhibitory factor and chicken ANP also appear to belong to the BNP group of natriuretic peptides and are further examples of species-specific processing of BNP compounds. Aldosterone inhibitory factor is a 35-amino acid peptide containing sequences identical with that found in porcine BNP-32 with three additional amino acid residues (Nguyen et al., 1989). It has been identified in chromaffin granules of bovine adrenal cells, a location that suggests that a paracrine mechanism may be important for regulating aldosterone secretion. Chicken ANP ($cANP_{1-29}$) isolated from the atrium and ventricles is also more closely related to porcine BNP than to mammalian ANP (Miyata et al., 1988) and is assumed to be a member of the BNP family (Toshimori et al., 1990; Akizuki et al., 1991).

3. C-type natriuretic peptide. The third member of the natriuretic hormone family is CNP, recently identified in porcine brain (Sudoh et al., 1990). CNP has a ring structure formed by an intramolecular disulfide bond as in both ANP and BNP, but it uniquely lacks the COOH-terminal extension from the ring; CNP terminates with the second cysteine residue that is critical for a 17-member ring structure. CNP was first isolated as a 22-amino acid peptide but has now been shown to be present mainly as CNP-53 in the brain (Minamino et al., 1991; Ueda et al., 1991). ProCNP may consist of 103 amino acids, although it has not yet been isolated in any species (Tawaragi et al., 1991). The amino acid sequences of CNP-22 are identical in the pig, rat, and human (Kojima et al., 1990; Tawaragi et al., 1990, 1991). Six nonmammalian natriuretic peptides, eel BNP-like peptide (Takei et al., 1990), killifish brain ANP (Price et al., 1990), frog CNP (Yoshihara et al., 1990), chicken CNP (Arimura et al., 1991), high molecular weight CNP from the heart of the European dogfish (Suzuki et al., 1991), and the shark heart CNP (Schofield et al., 1991) also lack the COOH-terminal tail sequence typical of ANP- and BNP-like peptides and are, thus, members of the group of CNPs. Of note, the extent of sequence homology of the eel BNP-like peptide is 86% to the porcine CNP (Takei et al., 1990), showing the highly conserved amino acid sequences during vertebrate evolution for CNPs.

CNP has pharmacological differences compared with either ANP and BNP, including a vigorous effect on smooth muscle but causing only a mild diuresis and natriuresis (Arimura et al., 1991). It has been shown to interact with a natriuretic peptide receptor distinct from

that of ANP and BNP (Sudoh et al., 1990; Koller et al., 1991; Ohyama et al., 1992). In anesthetized dog, CNP is a potent vasoactive peptide that in association with increases in plasma cGMP decreases arterial pressure, cardiac output, and atrial pressures (Stingo et al., 1992). In marked contrast to other natriuretic peptides such as ANP and BNP, CNP administration did not enhance sodium excretion or GFR (Stingo et al., 1992). Consistent with this minimal renal action of CNP is the observation by Furuya et al. (1990) that CNP does not increase cGMP production in isolated glomeruli despite its action to stimulate cGMP production in vascular smooth muscle. CNP also inhibits mitogenesis and proliferation of vascular smooth muscle cells through elevation of cGMP (Furuya et al., 1991, 1992) and is the most potent agonist of guanylate cyclase in endothelial cells from brain microvessels (Vigne and Frelin, 1992).

CNP appears to be a major ANP-like peptide existing in the brain regardless of species. In the human brain (hypothalamus and medulla-pons), the concentration of CNP is about 27- or 69-fold higher than that of ANP and BNP (Minamino et al., 1991). Rat CNP mRNA is specifically expressed in brain but not in peripheral tissues, including the cardiac atria and ventricles (Kojima et al., 1990; Ueda et al., 1991; Arimura et al., 1991). CNP immunoreactivity is also restricted to the central nervous system (Komatsu et al., 1991; Minamino et al., 1991), although small amounts of CNP-like immunoreactivity are also detected in the lower part of the gastrointestinal tract and kidney (Komatsu et al., 1991). Furthermore, in lower vertebrates, CNP is detected in the brain in extremely high concentrations (Takei et al., 1990; Yoshihara et al., 1990). Nevertheless, there seems to be a species difference in the organ that produces CNP, because a high molecular weight form (CNP-115) has recently been isolated from both cardiac atria and ventricles of the dogfish (Suzuki et al., 1991) and from the heart of spiny dogfish shark (Schofield et al., 1991). Thus, CNP may circulate as a hormone and have important biological functions in nonneuronal tissues. In support of this, CNP was shown to be a potent stimulator of chloride secretion in the isolated perfused rectal gland of the shark (Solomon et al., 1992).

III. Factors affecting Atrial Natriuretic Peptide Gene Expression

A variety of stimuli can modulate ANP gene expression (table 1). Usually, plasma and atrial IR-ANP content (e.g., μg/atrium) or concentration (e.g., ng/mg wet weight, μg/mg protein) have been measured to characterize changes in the synthesis, storage, and release of endogenous ANP in different experimental models. Because atrial IR-ANP concentration reflects ANP storage, which in turn is determined by the balance between rates of atrial ANP synthesis and release, measuring ANP mRNA levels by Northern and dot blot hybridizations as well as by in situ hybridizations provides a measure of synthetic activity. The quantity of ANP probe hybridization has been plotted as a function of 18S oligonucleotide or other control hybridization (β-actin, creatine kinase, etc.) or expressed relation to total RNA. Recently, these methods have also been applied to studies of ANP gene expression in humans with a variety of illnesses.

TABLE 1
Factors that affect ANP gene expression in vivo and in vitro

Pressure and volume overload
Patients with CHF and hypertension
Experimental models
Water deprivation
Salt intake
DOCA
Cardiomyopathic hamsters
Myocardial infarction
Pacing-induced heart failure
Aortocaval fistula
Aortic valve insufficiency
Spontaneous hypertension (SHR, SHR-SP)
Spontaneous biventricular hypertrophy in rats
Dahl salt-sensitive hypertension
DOCA/salt hypertension
Aortic constriction
Renovascular hypertension
Hypoxia
Monocrotaline-induced pulmonary hypertension
Vasopressin-deficient Brattleboro rats
Experimental cirrhosis
Diabetes mellitus
Nephrectomy
Hypophysectomy
Ureteral constriction
Pharmacological agents
Glucocorticoids
Thyroid hormones
Endothelin
Catecholamines (α_1-adrenergic agonists)
Growth factors (acidic fibroblast growth factor, basic fibroblast growth factor, transforming growth factor type β_1)
Products of arachidonic acid metabolism (PGE_2, $PGF_{2\alpha}$)

A. Atrial Atrial Natriuretic Peptide Gene Expression in Pressure and Volume Overload

1. Animal experiments. The first morphometric studies showed that the number of granules in the atriocytes decreases in animals fed a high salt diet or those treated with DOCA, whereas the number increases in animals deprived of water and sodium (Marie et al., 1976; de Bold, 1979). Subsequent studies have clearly demonstrated that ANP is a hormone whose release, storage, and synthesis in atrial cells are intimately linked to changes in intravascular volume and blood pressure. Most experimental models at the established stage of cardiac overload are characterized by increased circulating plasma ANP levels, decreased storage, and stimulation of cardiac ANP synthesis. The probable mediator of all these effects is atrial stretch.

a. CHANGES IN BLOOD VOLUME AND SODIUM BALANCE.

Chronic alterations of dietary salt and water balance have been shown to alter levels of IR-ANP and mRNA in atrial myocytes. Typically, water and sodium deprivation decrease both IR-ANP in plasma and ANP mRNA levels in atrial cells but increase IR-ANP in atriocytes. In contrast, volume-expanded animals, such as animals receiving a high salt diet, have decreased atriocyte ANP stores and increased ANP mRNA levels. Nakayama et al. (1984) first showed the effect of water deprivation on the level of atrial ANP mRNA measured by blot hybridization analysis in rats deprived of water for 2 and 4 days. ANP mRNA levels decreased when the animals were deprived of water, and the total content of the mRNA was about one-half to one-third of that in the control rats after 2 and 4 days of water deprivation, suggesting that blood volume may be an important factor in the regulation of ANP gene expression. Takayanagi et al. (1985) showed that 5 days of water or sodium deprivation in normal rats led to a 30 to 70% decrease in ANP mRNA and that this returned to control levels after administration of a 1.8% NaCl solution for 2 to 10 days. The ANP mRNA levels did not differ between the right and left atria. The atrial ANP mRNA level correlated positively with plasma IR-ANP and inversely with atrial content of IR-ANP. In volume-depleted rats (5 days), a 28% decrease was seen in the relative concentrations of atrial ANP mRNA compared with that of controls, whereas no statistically significant differences were seen in atrial levels of IR-ANP (Zisfein et al., 1986). Supporting these studies, ANP mRNA levels were lower in the low compared with the high sodium state in normal rats (Iwao et al., 1988), and water deprivation (3 days) increased atrial ANP stores (Schwartz et al., 1986; Ogawa et al., 1987). Thus, ANP mRNA transcription in atrial cardiocytes is inhibited under conditions of volume depletion associated with, at most, only a slight increase in the levels of stored IR-ANP; this high atrial content of ANP is the result of the decreased demand associated with the decreased synthesis of ANP.

Chronic feeding of a high salt diet or volume expansion cause opposite alterations in ANP synthesis, storage, and release to those observed after water and salt deprivation, although many conflicting results have also been published. A high salt diet (8.0% saline chow) for 2 weeks decreased right atrial IR-ANP content in normal rats (Schwartz et al., 1986). The response of the Dahl-S and Dahl-R rats to changes in salt and water balance was similar to the response of normal rats (Snajdar and Rapp, 1985; Schwartz et al., 1986). The humoral response of right atrial myocytes to sodium load appears to be more pronounced than that of left atrial myocytes, because Lattion et al. (1988) found that right atrial ANP mRNA levels in rats are enhanced after 1, but not 3, weeks of a high sodium (1%) intake. Also, dissociation between right and left atrial granulation was reported by Marie et al. (1976), who found that, after sodium loading, the index of granularity decreased in the right, but not in the left, atrium. In two short-term models (2 days) of volume overloading in rats, some undergoing binephrectomy and others ureteral ligation, an increase (38 to 46% compared to sham-operated rats) was seen in ANP mRNA content in left atrial myocytes (Lattion et al., 1986). Several other studies have also shown that long-term manipulation of dietary salt and water intake affect atrial ANP stores or synthesis similarly (Cantin et al., 1984; Pollock and Banks, 1984; Garcia et al., 1985a; Johnson, 1985; Kato et al., 1986b; Luft et al., 1986b; Pettersson et al., 1986; Gauquelin et al., 1988; Toshimori et al., 1988b; Hong et al., 1990; John and Morich, 1990; Lattion et al., 1990; Penner et al., 1990; Korytkowski and Ladenson, 1991). To summarize, animals given a high salt diet or that are volume expanded have increased ANP mRNA levels in atrial myocytes, whereas the amount of ANP appears to decrease in the atriocytes; the decreased atrial content of ANP in chronic volume overload thus reflects a state of increased ANP release which is not fully compensated by the increase in ANP synthesis. Furthermore, atrial ANP content appears to be more dependent on ANP release than on biosynthesis.

In contrast to above-mentioned studies, atrial ANP stores have also been reported to remain unaltered (Sugimoto et al., 1986; Iwao et al., 1988; Gauquelin et al., 1988; Jin et al., 1988a; Petersen et al., 1988) or even to increase in response to chronic volume or sodium overload (Hirata et al., 1984a; Tanaka et al., 1984; Morii et al., 1986b). For example, atrial IR-ANP concentration increased 69 to 80% after administration of a 1% NaCl solution to rats for 2 weeks (Tanaka et al., 1984), and no significant induction of atrial ANP mRNA compared with controls was detected after administration of a 1.8% NaCl solution for 2 weeks (Takaynagi et al., 1985). In addition, atrial IR-ANP values were lower in water-deprived rats (Gauquelin et al., 1988), water restriction decreased the natriuretic and diuretic activity of the atrium (Thibault et al., 1983), and ANP mRNA levels were reported to even increase after 2 to 4 days of dehydration (Hong et al., 1990). The discrepancies between these results may be due to variabilities between radioimmunoassays (see section IV.A), methodological differences (duration of treatment), or variations in the changes in intravascular volume and blood pressure in different experimental protocols.

b. DESOXYCORTICOSTERONE ACETATE. The finding that DOCA increased ANP mRNA levels was one of the first demonstrations of the regulation of ANP by hormonal factors. When animals and humans are given mineralocorticoids during a period of several days, there is a transient decrease in urinary sodium excretion resulting in Na^+ retention. In spite of continued mineralocorticoid administration, counterregulatory mechanisms come into play that return urinary sodium excretion to initial levels. This increase in urinary excretion

has been called escape. Several groups, although not all (Luft et al., 1986b), have shown that plasma ANP levels in rats, dogs, pigs, and humans increase in a time frame that coincides with escape from the mineralocorticoid effect (Ballermann et al., 1986; Grekin et al., 1986; Granger et al., 1987; Metzler et al., 1987; Zimmerman et al., 1987a). In addition, ANP mRNA levels of atrial myocytes in rats increased significantly (69%) as early as 12 h after a single depot injection of DOCA and remained elevated at 24, 48, and 72 h, indicating a rapid and sustained increase in ANP biosynthesis (Ballermann et al., 1986). In conscious dogs, compared with untreated control dogs, chronic DOCA administration increased ANP mRNA by 3.9-fold in the left atrium and 6.7-fold in the right atrium (Metzler et al., 1987). Thus, mineralocorticoid administration in the rat stimulates atrial myocyte ANP synthesis and release, which suggests a potential role for ANP in overriding mineralocorticoid-induced renal sodium retention. Whether the increase in ANP mRNA levels results from alterations in atrial stretch associated with extracellular volume expansion, from direct stimulation of ANP gene expression by DOCA, or as the result of other mechanisms remains to be established. Yet, because mineralocorticoids are known to signal new protein synthesis in some target tissues by interacting with nuclear receptors, a direct stimulation of ANP gene transcription could be involved in mediating the ANP gene expression.

c. EXPERIMENTAL MODELS OF CONGESTIVE HEART FAILURE. Several models of cardiovascular pathophysiology also exhibit abnormalities in stored levels of both atrial IR-ANP and ANP mRNA. The cardiomyopathic hamster has been found to be a useful model for CHF, and plasma levels of IR-ANP are markedly elevated in animals with moderate or severe heart failure (Edwards et al., 1986a, 1988a; Franch et al., 1986, 1988; Ding et al., 1987; Cantin et al., 1988; Thibault et al., 1989c; Wong et al., 1992). At the ultrastructural level, atrial cardiocytes from cardiomyopathic hamsters with severe heart failure show intense secretory stimulation characterized by reduced atrial ANP granularity (Edwards et al., 1986a; Ding et al., 1987; Cantin et al., 1988, 1990).

Most investigators also now agree that the amount of IR-ANP decreases significantly in both the left and right atrium during CHF in these animals. Chimoskey et al. (1984) and Dlouha and McBroom (1986) reported that the atrial content of ANP measured by bioassay is reduced in this animal model of CHF. Atrial tissue from cardiomyopathic hamsters contains significantly less IR-ANP (50 to 80%) than that from control animals (Franch et al., 1986, 1988; Ding et al., 1987; Edwards et al., 1988a; Cantin et al., 1988; Thibault et al., 1989c; Wong et al., 1992). The results reported by Currie et al. (1987) differ from those of others; they found a 2.2-fold greater total atrial IR-ANP content in the cardiomyopathic hamsters. Because cardiomyopathic hamsters show a significant elevation of left and right ventricular end-diastolic pressure, the decrease in atrial IR-ANP is probably related to the increased pressure leading to increased stretch and enhanced secretion from cardiocytes. The striking reduction in IR-ANP in these animals shows that the synthesis rate does not keep pace. Indeed, although a preliminary study suggested a 3.4-fold increase in ANP mRNA levels (Currie et al., 1987), the atrial levels of ANP mRNA in cardiomyopathic hamsters gradually decreased (20 to 40%, pooled data) as the CHF became more severe (Thibault et al., 1989c) or were not significantly different from those in normal hamsters (Franch et al., 1988). Thus, these findings support the suggestion that ANP gene expression in maximally stimulated atrial cells does not increase significantly to compensate for the increased peptide demand in CHF, and this leads to depletion of atrial ANP stores.

The rat coronary artery ligation model of heart failure has been shown to produce varying degrees of left ventricular dysfunction in proportion to infarct size. Rats with large infarcts have elevations in left ventricular filling pressures and ventricular dilation. The rats with myocardial infarction and heart failure also have fluid retention, inability to excrete a sodium load, and vasoconstriction similar to those seen in human CHF. Rector et al. (1985) determined the atrial ANP content in rats with chronic CHF (3 months after myocardial infarction produced by coronary artery ligation). These rats had reduced ANP activity within the atria compared with sham-operated rats measured by bioassay. As in the hereditary cardiomyopathic hamster model, plasma ANP levels are significantly increased in rats with myocardial infarction (Tsunoda et al., 1986; Drexler et al., 1987; Lee et al., 1987; Mendez et al., 1987; Riegger et al., 1987; Tikkanen et al., 1987a,b; Awazu et al., 1989; Bilder et al., 1989a). IR-ANP concentrations are lower in both the right and left atria (Tsunoda et al., 1986; Mendez et al., 1987; Drexler et al., 1989) or right atria (Bilder et al., 1989a) compared with controls. Thus, atrial ANP stores in this rat model of heart failure are depleted. The ANP mRNA concentration (related to 18S ribosomal RNA) was similar in both left and right atrial myocytes in rats with large infarcts compared with control rats. Yet, an overall increase in atrial ANP synthesis (ANP mRNA content) was noted in rats with large infarcts (Mendez et al., 1987). Similarly, the atrial ANP mRNA content was increased by 38% only in rats with large infarcts compared with sham-operated rats, whereas the ratio of atrial ANP mRNA to β-actin was not significantly increased (Drexler et al., 1989). Michel et al. (1988) found a 4.9- and 7.2-fold increase in the right and left atrial ANP mRNA contents, respectively, 2 months after myocardial infarction. Treatment with perindopril, an ACE inhibitor, partially reversed this ANP gene expression. These results show that activation of the ANP system is associated with depleted atrial ANP stores, and the de-

pletion correlates with the severity of the heart failure and the increase in intracardiac pressures. In rats with myocardial infarction, elevated circulating ANP levels are maintained through enhanced atrial synthesis and release, but the increase in atrial ANP gene expression seems to be limited, suggesting that the ANP gene may be expressed at near its maximal rate in normal atria.

Plasma IR-ANP concentrations are also increased in dogs and sheep with CHF because of rapid right ventricular pacing (Riegger et al., 1988; Moe et al., 1989; Redfield et al., 1989b; Perrella et al., 1992). Armstrong et al. (1986) showed that the natriuretic activity from the right atrium of dogs with paced ventricles is significantly less than that from controls, and ultrastructural changes in the atrial cells indicate extreme stimulation of the secretory apparatus of ANP (Riegger et al., 1988). After 1 week of pacing, ANP release is attenuated with a reduction of left atrial IR-ANP concentration (Moe et al., 1991). In support of these studies, right atrial tissue IR-ANP concentrations decreased, and atrial ANP mRNA levels increased with the progression of CHF produced by rapid ventricular pacing in dogs (Perrella et al., 1992). Circulating ANP levels were unchanged in an animal model of acute, low-output heart failure produced by inflation of a balloon in the inferior vena cava (Redfield et al., 1989a). On the other hand, in dogs and rats with arteriovenous fistulas and the syndrome of chronic high-output heart failure, long-term increases in atrial pressure appear to be a sustained stimulus for the release of ANP (Villarreal et al., 1987; Hoffman et al., 1988; Winaver et al., 1988; Garcia et al., 1990a). A slight increase (26%) in ANP mRNA content of right atria could also be detected after 2 weeks in this experimental model of heart failure (Lattion et al., 1986). Increased plasma IR-ANP levels, decreased atrial concentration of IR-ANP, and unchanged atrial ANP mRNA concentration (in relation to β-actin mRNA) were found in rats with experimental aortic valve insufficiency produced by a polyethylene rod (Morita et al., 1990).

Field (1988) generated transgenic mice that carry fusions between the transcriptional regulatory sequences of ANP and those encoding SV40 T-antigen. Although both atria express the fusion gene (*ANP-Tag*), the pathological response develops asymmetrically; the right atrium undergoes a several hundred-fold increase in mass, whereas the left atrium remains relatively normal in size (Field, 1988; Steinhelper and Field, 1990). Hyperplasia is accompanied by features associated with cardiac failure, including increased plasma creatine kinase activity and ventricular dysrhythmias. The atrial tumor tissue can proliferate over protracted periods at ectopic sites without losing a variety of characteristic cardiac phenotypes, as determined by diverse functional and structural criteria (Steinhelper et al. 1990b; Steinhelper and Field, 1990; Delcarpio et al., 1991). Furthermore, cultured cardiomyocytes derived from the transplantable line (AT-1 cells) retained a pattern of gene expression characteristic of normal adult mouse atrial myocytes (Lanson et al., 1992). AT-1 cells also synthesized, stored, and secreted ANP in a manner similar to nontransgenic atrial myocytes (Lanson et al., 1992). These cells could, therefore, serve as a source for the study ANP synthesis, processing, and release.

Transgenic mice harboring a chimeric gene linking the 5′-flanking sequences of a mouse protamine 1 gene to the coding sequence of the SV40 T-antigen also develop spontaneous rhabdomyosarcomas of the right atria (Behringer et al., 1988). The presence of the tumors is accompanied by marked increases in plasma IR-ANP concentrations (Gardner et al., 1992). The IR-ANP and ANP mRNA transcripts present in the tumors appear to be similar in size to those found in normal mouse atria, although absolute levels of expression varied among different cells in the population (Gardner et al., 1992). Recently, a cell line (MCM1) with cardiac properties that originated from cells isolated from a SV40-induced tumor in the right atria of a transgenic mouse was described (Sculptoreanu et al., 1992). Thus, these tumors and the cell line also represent a potential model for the study of ANP biosynthesis and secretion.

d. EXPERIMENTAL MODELS OF HYPERTENSION. The SHR and its substrain, SHR-SP, are genetically hypertensive rat strains that have been extensively studied in hypertension research. SHR slowly develop a progressive increase in arterial pressure compared with rats in which hypertension is induced by surgical intervention or pharmacological means. This makes them a suitable model for studying the sequential alterations associated with pressure overload. By the time SHR and SHR-SP have established hypertension, they have significantly increased plasma IR-ANP concentrations (for references, see Arai et al., 1988; Ruskoaho and Leppäluoto 1988a), and this increased secretion may be a compensatory response to hypertension. Studies of the biosynthesis and secretion of ANP in these hypertensive rat models will improve our understanding of the regulation of ANP gene expression in pressure overload.

No obvious strain differences in ANP gene expression were noted between SHR and WKY rat fetal hearts (Scott and Jennes, 1987, 1988). Sonnenberg et al. (1983) first examined the natriuretic activity in atrial extracts prepared from adult rats and found that the natriuretic activity was reduced in 16- to 17-week-old SHR rats compared with age-matched control WKY rats. Subsequent measurements of atrial IR-ANP levels in SHR have produced varying results; some investigators have reported reduced atrial IR-ANP concentrations in SHR (Higa et al., 1985; Imada et al., 1985; Gutkowska et al., 1986a; Morii et al., 1986a,c; Arai et al., 1987; de Leon et al., 1987; Kohno et al., 1988, 1989, 1990b; Gu and Gonzalez-Lavin, 1988; Garcia et al., 1989), and others have found no difference between SHR and the normotensive WKY rats (Garcia et al., 1985c, 1989; Gutkowska et al.,

1986a; Arai et al., 1988; Jin et al., 1988a; Ruskoaho and Leppäluoto 1988a; Lachance and Garcia, 1991a,b); some have even reported an increase in atrial IR-ANP levels (Winquist et al., 1984; Gutkowska et al., 1986a; Xie et al., 1986; Haass et al., 1987a; Kato et al., 1987; Garcia et al., 1989; Komatsu et al., 1992). Long-term antihypertensive treatment with diltiazem (Kohno et al., 1988) or methyldopa (Kohno et al., 1990b) reduced blood pressure and cardiac hypertrophy and consistently increased left atrial IR-ANP concentration in SHR, suggesting that atrial ANP synthesis can be reversed by eliminating pressure overload. Yet, ACE inhibitors (CS622, captopril for 10 days) and hydralazine had no effect on atrial IR-ANP concentration in SHR (Oda et al., 1988; Kohno et al., 1990b; Lachance and Garcia, 1991c).

To assess the biosynthesis of ANP in the atria, ANP mRNA levels have been measured in SHR and WKY rats. Concentrations and total contents of atrial ANP mRNA and IR-ANP were similar in SHR and SHR-SP rats at 27 weeks of age when the SHR strains have established hypertension compared to those of WKY (Arai et al., 1988). Similarly, no apparent differences were noted in the right and left atrial ANP mRNA levels and IR-ANP concentrations between 1-year-old (Ruskoaho et al., 1989a; Kinnunen et al., 1990) or 17-week-old (Komatsu et al., 1992) SHR and WKY rats. In a recent study, atrial ANP mRNA and IR-ANP levels were measured from 2- and 21-month-old SHR and WKY rats (Kinnunen et al., 1991). Atrial IR-ANP concentrations and ANP mRNA levels (related to 18S ribosomal RNA) increased with age in the normotensive rats; the IR-ANP concentration was 74%, and the ANP mRNA level was 90% greater in the atria from the 21-month-old WKY rats compared to the 2-month-old animals. In contrast, atrial ANP concentrations in the SHR decreased significantly (35%) with increasing age, and ANP mRNA levels were similar in young and old SHR (Kinnunen et al., 1991). Furthermore, the atrial ANP mRNA level in SHR did not differ significantly from the age-matched WKY rats at either age.

These results show that, as hypertension progresses in SHR, atrial ANP stores are depleted and plasma IR-ANP concentrations increase compared with their respective control animals. The decrease in atrial ANP is more pronounced in the left than in the right atrium (Imada et al., 1985; Gutkowska et al., 1986a; Morii et al., 1986c). However, the degree of activation of the ANP gene in the atria in vivo in response to chronic cardiac overload is limited. In fact, hypertension in SHR may be accompanied by a lower ability of atrial myocytes to synthesize ANP in response to increased blood pressure.

Dahl-S rats develop hypertension when fed a high salt diet, whereas Dahl-R rats do not, making them important genetic models in which to study ANP gene expression and its relationship to salt-induced hypertension. Plasma IR-ANP levels in young Dahl-S rats are similar to those in young Dahl-R rats but increase as blood pressure increases in older animals (Snajdar and Rapp, 1986). In contrast to SHR hypertension, IR-ANP concentrations in atrial tissue are higher in Dahl-S rats than in Dahl-R rats when measured by both bioassay (Hirata et al., 1984a; Snajdar and Rapp, 1985) and immunoassay (Schwartz et al., 1986; Snajdar et al., 1986, 1987; Wilson et al., 1988; John and Morich, 1990), although conflicting results have been also published (Gutkowska et al., 1986b; Tanaka and Inagami, 1986b; Sterzel et al., 1987). This strain difference in atrial tissue IR-ANP is not present between 1 and 15 days of age but develops by the age of 1 month (Snajdar et al., 1986; Wilson et al., 1988) and persists up to the age of 8.5 months (Dene and Rapp, 1987; Rapp and Dene, 1988).

It is not known why the ANP level is high in the atrial tissue of the Dahl-S rat. Theoretically, it could be due to increased synthesis and storage of ANP or impairment of its release. It seems unlikely that atrial ANP synthesis is increased in Dahl-S rats because ANP mRNA is present in similar amounts in the atria of Dahl-S and Dahl-R rats from 1.5 to 8.5 months of age, although ANP mRNA decreases with increasing age in both strains (Dene and Rapp, 1987). Onwochei and Rapp (1989) reported data obtained from isolated heart-lung preparations that support the concept that Dahl-S rats have impaired ANP release, i.e., Dahl-S rats release less ANP than Dahl-R rats as atrial pressure changes. Similarly, in one study in which 8-week-old Dahl-S and Dahl-R rats were challenged with an acute i.v. saline load, Dahl-R rats showed a 50% greater increase in plasma IR-ANP concentration than Dahl-S rats, although the difference was not statistically significant (Sterzel et al., 1987). These studies give no information concerning the potential mechanism whereby hearts from young Dahl-S rats release less ANP, but this may involve genetically mediated defective ANP release from the heart. Thus, in contrast to ANP concentrations in SHR, atrial ANP concentrations are increased with plasma ANP levels in Dahl-S hypertensive rats, whereas atrial ANP mRNA levels remain unchanged.

DOCA/salt hypertension is generally accepted to be a model of volume-expanded hypertension. Ackermann and Irizawa (1984) found that fucose, a glycoprotein precursor, was incorporated more rapidly into the atria of DOCA/salt-treated rats compared to sodium-deprived control rats, which suggested increased protein synthesis in the treated group. Early studies of ANP content in DOCA/salt-treated animals (measured by bioassay) produced conflicting results; atrial extracts from DOCA/salt-treated rats (3 weeks) showed a significantly more potent natriuretic and diuretic effect than extracts from sodium-deficient rats (Ackermann and Irizawa, 1984). Decreased atrial ANP content with elevated plasma ANP levels, measured by immunoassay, in response to chronic treatment of DOCA/salt in rats has been reported in this

model (Sugimoto et al., 1986;, Sato et al., 1990), although others (Garcia et al., 1987d;, Itoh et al., 1991) found no differences, probably because of a shorter follow-up period. Three weeks of DOCA/salt treatment of rats increased ANP mRNA content both in the right (53%) and left (91%) atria (Lattion et al., 1986). Similarly, the level of ANP mRNA in the atria of DOCA-treated rats was 2-fold higher than that of control rats (Itoh et al., 1991). Using a quantitative in situ hybridization method, Nunez et al. (1989) found that 4 weeks of DOCA/salt treatment of rats elevates right and left atrial ANP mRNA levels by 72%, whereas no difference was seen between ANP mRNA probe hybridization to left and right atria.

Other models of nonspontaneous experimental hypertension have received less attention. Constriction of the abdominal aorta increases cardiac afterload and causes left ventricular hypertrophy that is proportional to the degree of constriction. Aortic banding in rats caused a significant decrease in IR-ANP concentration in left and right atria as well as increased plasma IR-ANP levels (Day et al., 1987; Mercadier et al., 1989). In rats with coarctations, ANP immunoreactivity detected by light and electron microscopic immunocytochemistry was decreased, and in rats whose coarctations were released, the intensity of ANP immunostaining returned to the control level (Gu et al., 1989). The level of ANP mRNA in atria determined by in situ hybridization remained largely unchanged during coarctation and after coarctation release (Gu et al., 1989).

Among other models of nonspontaneous experimental hypertension, the one-kidney, one-clip rat has also been shown to possess low atrial IR-ANP levels (Garcia et al., 1985b, 1987a,d, 1988). The increased plasma IR-ANP levels at weeks 1 and 2 were accompanied by a lower total atrial IR-ANP content and concentration in the left but not in the right atrium, whereas low atrial IR-ANP concentrations in both atria were noted 8 weeks after nephrectomy (Garcia et al., 1987a,d). After unclipping (13 days), there were no differences in either IR-ANP content or concentration between the normotensive and the hypertensive group. Matsubara et al. (1990b) assessed the changes in ANP and its mRNA in atria in relation to hemodynamic factors during antihypertensive treatments in two-kidney, one-clip renovascular hypertensive rats. A small increase (80%) in the ANP mRNA content of left atria, no increase in mRNA levels in the right atria, and a significant decrease in IR-ANP levels in both atria were found, suggesting stimulated ANP synthesis and an enhanced rate of atrial ANP secretion in these rats. The increased ANP synthesis in left atria returned to the control level after enalapril treatment or removal of the clipped kidney but not after hydralazine treatment (Matsubara et al., 1990b). This suggests that atrial ANP synthesis can be reversed by removing causative hypertensinogenic stimuli. In contrast, no alterations were noted in atrial IR-ANP concentrations in two-kidney, one-clip hypertensive animals (Garcia et al., 1986b, 1987c,d). Garcia et al. (1987d) also studied ANP storage in other models of experimental hypertension and found that the atrial IR-ANP concentration was lower in the left atrium of rats with adrenal regeneration hypertension. Taken together, in pressure-dependent hypertension, chronic atrial wall distension leads to increased plasma IR-ANP levels and preferentially decreases left atrial ANP stores.

Several lines of evidence suggest that ANP may be involved in the pathogenesis of pulmonary hypertension. Hypoxia is a potent pulmonary vasoconstrictor and chronic exposure leads to sustained pulmonary hypertension and the development of right ventricular hypertrophy. In rats exposed to hypoxia for 21 days, the right atrial IR-ANP concentration decreased (27%) and plasma IR-ANP concentration increased compared with control animals breathing air, whereas left atrial IR-ANP concentration increased (68%) significantly (McKenzie et al., 1986). Accordingly, Stockmann et al. (1988) found that 3 weeks of hypoxia resulted in a 50% decrease in right atrial IR-ANP and ANP mRNA levels did not differ from normoxic controls. In the left atrium of hypoxic rats, ANP mRNA decreased by 33% without a significant change in IR-ANP (Stockmann et al., 1988). Depletion of right atrial IR-ANP levels and the reduction in electron-dense granules with an increase in plasma IR-ANP levels has been confirmed by others (Winter et al., 1989). Thus, chronic hypoxia with pressure overload of the right heart and decreased venous return to the left heart stimulates ANP release from the right atrium and inhibits ANP synthesis and release from the left atrium.

A reduction in atrial IR-ANP with elevated circulating peptide levels has also been demonstrated in another model of chronic pulmonary hypertension induced by monocrotaline (Akimoto et al., 1988b; Ceconi et al., 1989; Oehlenschlager et al., 1989; Agnoletti et al., 1990a). This reduction of IR-ANP was more marked in the atrium under greater mechanical stress (Ceconi et al., 1989). Hybridization analysis of total RNA extracted from atrial tissue indicated that atrial ANP mRNA content was increased in monocrotaline-treated rats (Oehlenschlager et al., 1989). No significant difference in tissue levels of IR-ANP was noted in the left atrium between monocrotaline-treated and untreated rats (Akimoto et al., 1988b). Thus, chronic distension of the right atrium caused by either hypoxia or monocrotaline injection induces ANP secretion from the right atrium and, consequently, storage depletion.

e. OTHER EXPERIMENTAL MODELS. Brattleboro (DI) rats, homozygous for hereditary genetic hypothalamic diabetes insipidus, exhibit the following: (*a*) a large urinary output and water intake, (*b*) a high plasma sodium and osmolality, and (*c*) a lack of pituitary vasopressin. Thus, they provide a suitable model for investigating the

regulation of ANP synthesis and release. Baseline plasma IR-ANP levels do not differ in DI and control rats (Ogawa et al., 1986, 1987; Lavigne et al., 1988; Sakata et al., 1988b; Ruskoaho et al., 1989b), although a 45% increase in plasma ANP was observed recently (Bahner et al., 1990). Both right and left atrial IR-ANP content (Ruskoaho et al., 1989b) or the right (Ruskoaho et al., 1989b) and left (Lavigne et al., 1988) auricular IR-ANP concentration have been shown to be higher in DI than in control animals. Atrial IR-ANP content in DI rats measured by bioassay has been reported to be increased (Johnson, 1985). The increase in ANP mRNA levels was greater in the left atrium than in the right atrium of the Brattleboro rats (Lavigne et al., 1988; Ruskoaho et al., 1989b). These findings of normal or slightly elevated plasma IR-ANP levels with increased ANP synthesis and storage in the atria of Brattleboro rats suggest that local synthetic mechanisms are altered in the atrial tissue of DI rats. The relationship of moderately increased blood pressure, activation of the renin-ANG-aldosterone system, and the elevation of plasma sodium and osmolality or other factors that affect ANP release and content to increase atrial ANP synthesis remains to be studied.

Patients and experimental animals with cirrhosis of the liver often show impairment of renal function resulting in water and sodium retention with ascites and edema formation. ANP levels in those with cirrhosis have varied, and conflicting results have been seen in studies of rats with experimental liver cirrhosis and in humans (for reviews, see Epstein, 1989; Cody, 1990). In dogs with chronic bile duct ligation, Maher et al. (1990) demonstrated a triphasic response of plasma IR-ANP levels following ligation. In response to common bile duct ligation, plasma peptide levels (at 2 weeks) decreased by almost 50%. Then, by the 5th and 6th postoperative weeks, plasma IR-ANP levels began to increase. Finally, during the ascitic phase of cirrhosis, plasma IR-ANP concentration again decreased (Maher et al., 1990). Thus, both the phase and probably the experimental model of liver cirrhosis may explain the reported differences in plasma IR-ANP levels. In one study, atrial extracts from cirrhotic rats with ascites showed a significantly lower diuretic and natriuretic activity than did atrial extracts from control animals (Jimenez et al., 1986), suggesting that the atrial ANP content is reduced in these animals. In contrast, IR-ANP content has been reported to remain unchanged following bile duct ligation (Maher et al., 1990).

Diabetes mellitus is also associated with abnormalities in fluid and electrolyte balance and cardiac function, and the effects streptozotocin-induced diabetes on ANP gene expression have been recently studied in SHR and WKY rats (Matsubara et al., 1990a). The diabetic SHR exhibited a 36% increase in left atrial ANP mRNA levels (relative to β-actin mRNA) after 4 and at 8 weeks, this accumulation increased by 44% in the left atria and by 27% in the right atria. On the other hand, atrial IR-ANP levels in the diabetic SHR decreased by 42% in left atria at 4 weeks and by 43% in both left and right atria at 8 weeks (Matsubara et al., 1990a). The increased ANP synthesis was related to elevated intraventricular filling pressure and was reversed by insulin therapy (Matsubara et al., 1990a). Results of these studies suggest that the ANP system in animals with diabetes mellitus may function as a cardiac compensatory mechanism against elevation of intraventricular pressure. In contrast to the above findings in drug-induced diabetes models, plasma IR-ANP levels were decreased with an increase in right atrial IR-ANP concentration and atrial specific granules in nonobese spontaneously diabetic mice (Yano et al., 1991). These changes resemble those observed after volume depletion, and in fact, hyperglycemic mice were profoundly dehydrated (Yano et al., 1991). One preliminary study also showed that the atria of mice made obese by gold thiolglucose contain more ANP than those of control mice (Iida et al., 1990).

Other procedures reported to affect ANP synthesis and storage include sympathectomy and nephrectomy. Immunohistochemical analysis of left atria showed that the intensity of staining is reduced in denervated canine hearts (Kaczmarczyk et al., 1987). On the other hand, chemical sympathectomy of the rat heart by 6-hydroxydopamine after 5 days was associated with significant increases in the density of ANP-storing granules and IR-ANP concentration (Albino-Teixeira et al., 1990). Atrial ANP content measured by bioassay was decreased in five of six nephrectomized rats with chronic renal failure (Petersen et al., 1988). Similarly, atrial IR-ANP content decreased as the IR-ANP concentration increased in a remnant kidney model of progressive renal failure in the rat (Jackson et al., 1988). The diurnal rhythm may also influence atrial ANP stores, because the fluctuation in plasma IR-ANP concentration during 24 h was positively correlated to the numerical densities of secretory granules (Watanabe and Uchiyama, 1988). Furthermore, long-term endurance physical training increased plasma IR-ANP levels, whereas right and left atrial IR-ANP levels did not differ significantly between control and trained rats; however, a significant negative correlation between circulating and left atrial IR-ANP concentration was found (Ibanez et al., 1990). Cardiac ANP content measured by bioassay was also similar in pregnant and nonpregnant rats (Kristensen et al., 1986).

Changes in atrial ANP synthesis and storage have been described in response to chronic calcium and magnesium feeding as well as long-term drug infusions. A high calcium (Kohno et al., 1989; Wong et al., 1991c) diet decreased atrial IR-ANP concentration with a significant increase in plasma IR-ANP levels and increased IR-ANP release from isolated perfused hearts (Wong et al., 1991c). A high magnesium diet increased atrial IR-

ANP concentration with a significant increase in plasma IR-ANP levels and increased IR-ANP release from isolated perfused hearts (Wong et al., 1991b), suggesting that magnesium ions may affect the synthesis of ANP. Infusion of AVP and a V_2-agonist, deamino-8-D-arginine vasopressin, into Brattleboro rats, by expanding their extracellular volume, significantly reduced atrial IR-ANP content while increasing plasma IR-ANP levels (Ogawa et al., 1987), further showing that atrial stores reflect secretion rather than biosynthesis of ANP. Accordingly, chronic ANG II infusion for 7 days increased plasma IR-ANP levels with lower right and left atrial IR-ANP concentrations (Gauquelin et al., 1991). Furthermore, atrial content of IR-ANP was higher in the right atria than in left atria which was associated with normal plasma IR-ANP levels in the phenylephrine- and epinephrine-treated groups, when both agents were infused s.c. by osmotic minipumps for 5 days (Garcia et al., 1986a). Infusion of isoproterenol for 7 days in rats also caused a decrease of atrial content of IR-ANP with increased plasma IR-ANP levels (Agnoletti et al., 1990b). Superfused atria isolated from animals in which isoproterenol was infused showed reduced basal release of IR-ANP, and they did not respond to stretch or isoproterenol (Agnoletti et al., 1990b). In another study, however, isoprenaline and carbachol which significantly increased plasma levels had no effect on atrial IR-ANP levels (Garcia et al., 1986a).

Finally, results of studies of ANP gene expression in perinatal rats suggest that hemodynamic or other stimuli influence ANP biosynthesis and storage. Ventricular ANP gene expression appears to peak soon after birth; ventricular ANP mRNA levels are extremely high in the neonate and approach the levels seen in adult atria (Wei et al., 1987b; Wu et al., 1988). This peak may reflect the dramatic changes in central hemodynamics that occur at partus. Consistently, plasma IR-ANP levels were extremely high on day 1 after birth (Wu et al., 1988), and there was a significant nadir in the concentration of IR-ANP in the right and left atria on the day before birth that persisted for the first 48 h of life (Dolan et al., 1989). Repletion of ANP stores begins in the right atrium by day 2 and in the left atrium between days 2 and 5. These changes in ANP synthesis, storage, and release resemble those in normal adult rat hearts in response to the severe pressure and volume overload already described.

2. *Human studies.* Patients with CHF have been shown to have high plasma concentrations of IR-ANP (Tikkanen et al., 1985a; Burnett et al., 1986; Raine et al., 1986), and human atria contain increased total IR-ANP concentrations in severe CHF. In a preliminary study, an extremely high tissue IR-ANP content in the atria of patients with combined mitral valve stenosis and insufficiency was found. Those patients with a large number of cardiac autophagolytic endocrine cells had a poorer outcome (Späth et al., 1986). In another preliminary study, atrial ANP mRNA concentrations in a patient with DCM were similar to those in the hearts without cardiac complications (Saito et al., 1987b). However, the total atrial ANP mRNA content (3-fold difference), as well as IR-ANP content and concentration, was higher in the DCM atrium (Saito et al., 1987b). Other studies have shown that the IR-ANP concentration in the right atrium of patients with a variety of heart diseases increases with the severity of CHF (Akimoto et al., 1988c; Sugawara et al., 1988b,c; Saito et al., 1989a; Naruse et al., 1990). Two- to 3-fold increases in the IR-ANP and ANP mRNA levels were found in the atria from patients with DCM or myocardial infarct compared with the normal atrium (Saito et al., 1989a). Atrial IR-ANP concentrations were generally higher in patients with valvular heart diseases than in patients with ischemic heart disease or congenital heart disease (Sugawara et al., 1988c). Interestingly, the IR-ANP concentrations in atria from three patients with class IV severe CHF were lower (45%) than those in patients with class III CHF, suggesting possible decreases in ANP storage in the severest stage of chronic heart failure (Sugawara et al., 1988c). On the other hand, left and right atria from patients in the DCM group contained essentially similar amounts of IR-ANP as seen in the atria in the control group (Tsuchimochi et al., 1988).

ANP in the human auricle consists of three major ANP components, $proANP_{1-126}$, ANP_{99-126}, and β-ANP, a dimer of ANP_{99-126} (Kangawa et al., 1984a,b,c, 1985; Kangawa and Matsuo, 1984). The predominant component in mild CHF is $proANP_{1-126}$, whereas β-ANP and ANP_{99-126} prevail in severe CHF, and β-ANP, in particular, is increased in the failing human heart (Akimoto et al., 1988c; Sugawara et al., 1988b,c; Naruse et al., 1990). Thus, the posttranslational $proANP_{1-126}$ processing in the human heart is altered along with severity of CHF. The peptide can also form amyloid fibrils and cause pathological protein deposition in atria (Kaye et al., 1986; Johannsson et al., 1987; Linke et al., 1988; Pucci et al., 1991). In summary, the failing heart appears to augment synthesis and secretion of ANP as a compensatory response to CHF. Whereas ANP is depleted in the hearts of experimental animals with heart failure, it is not in the failing human heart, probably because of the ability of the failing heart to maintain ANP storage.

In two recent reports, the relationship among the levels of ANP mRNA and atrial storage and circulating plasma IR-ANP levels was studied in patients with heart failure (Haass et al., 1990, Fischer et al., 1991). Plasma ANP concentrations correlated with atrial ANP mRNA levels, as well as with pulmonary artery pressure and left atrial pressure (Haass et al., 1990; Fischer et al., 1991). The increase in ANP mRNA level was not accompanied by a simultaneous increase in β-actin expression, and the atrial ANP mRNA/β-actin ratio increased with left ventricular filling pressure (r = 0.80 to 0.91). Haass et al.

(1990) and Fischer et al. (1991), like others before (Wambach et al., 1987; Naruse et al., 1990), however, found no relationship between the right atrial IR-ANP content and the expression of ANP mRNA in the atria and the plasma IR-ANP levels. The expression of the ANP gene in the atria in the failing human heart thus appears to be related to left ventricular filling pressure.

B. Ventricular Atrial Natriuretic Peptide Gene Expression in Pressure and Volume Overload and Cardiac Hypertrophy

1. Animal experiments. Several models of acute and chronic volume overload in the rat induce ventricular ANP expression (table 1) as determined by changes in tissue ANP mRNA and IR-ANP concentrations and immunohistochemistry. Lattion et al. (1986) measured ANP mRNA levels in rat atrial and ventricular myocardia in four models of volume loading: binephrectomy (for 48 h), ligature of both ureters (for 48 h), DOCA/salt (for 3 weeks), and aortocaval fistula (for 2 weeks). These procedures produce marked changes in cardiovascular hemodynamics, and each experimental condition was accompanied by a highly significant increase in ANP mRNA content in the left ventricle visualized by in situ hybridization in the fistula model. The most striking induction of ventricular ANP expression occurred in the aortocaval fistula model, with an 11-fold increase in ANP mRNA and was associated with the most pronounced ventricular hypertrophy. In the DOCA/salt model with moderate myocardial hypertrophy, ANP mRNA increased 4-fold in the left but not in the right ventricle. In the right ventricle, the increase in ANP mRNA (3-fold) was only seen in the binephrectomy model (Lattion et al., 1986). In a later study, the ANP mRNA level in the ventricles of DOCA/salt rats exhibited a 7-fold increase compared with that of control rats (Itoh et al., 1991).

Augmented ventricular expression of ANP and the ANP gene has been found in several other animal models which include those with genetic and experimental hemodynamic loads. The SHR strain is also a particularly appropriate experimental model for studying alterations in ventricular hypertrophy. Unlike other models of cardiac hypertrophy, the condition is spontaneous, and the gradual and progressive increase in cardiac mass in response to chronically elevated blood pressure occurs as a function of age. Myocardial performance in SHR is impaired when ventricular hypertrophy is severe and heart failure develops. In SHR, these cardiovascular changes are now known to be associated with the reinduction of the ANP-coding gene.

Takayanagi et al. (1987) reported that the IR-ANP concentration of the apical portion of ventricles was 3-fold higher in the 15-week-old SHR than in the corresponding normotensive WKY rats, and others have confirmed the participation of the ventricle in ANP expression in SHR (Arai et al., 1988; Ruskoaho et al., 1989a; Kinnunen et al., 1990, 1991; Matsubara et al., 1990a; Komatsu et al., 1992). Ventricular ANP mRNA levels in 27-week-old SHR and SHR-SP with established hypertension and ventricular hypertrophy were markedly increased, and the total ventricular ANP mRNA contents in SHR and SHR-SP were approximately 50 and 250%, respectively, of the corresponding atrial contents, although it was only 6% in WKY rats (Arai et al., 1988). When ANP gene expression was studied in young and old hypertensive animals, the total amount of IR-ANP in the ventricles of the SHR increased 41-fold, and ANP mRNA levels increased 12.9-fold from the age of 2 to 21 months (Kinnunen et al., 1991). Total IR-ANP content in the ventricles of 21-month-old SHR was 5.4-fold and the IR-ANP concentration was 3.6-fold greater than in the WKY rats, although no differences were found in IR-ANP and ANP mRNA levels between the young WKY rats and SHR (Kinnunen et al., 1991). In SHR, treatment with minoxidil increased cardiac hypertrophy and produced a 2.1-fold increase in ventricular ANP mRNA compared with untreated controls (Kinnunen et al., 1990). Furthermore, a highly significant correlation was found between ventricular IR-ANP content and ventricular hypertrophy in SHR (Kinnunen et al., 1990), suggesting that the augmented expression of the ANP gene in the diseased ventricle is due to ventricular hypertrophy.

The localization of ANP-immunoreactive material in the specific-granules in the ventricles provides additional evidence for the increased ventricular ANP synthesis during spontaneous hypertension (Gu and Gonzalez-Lavin, 1988; Gu et al., 1991; Kinnunen et al., 1991). The ANP-positive cells were mainly distributed in the midmyocardium and subendocardium, whereas the epicardium was generally negative. However, Kinnunen et al. (1991) found moderate or weak immunoreaction in approximately two of three of the myocytes of old SHR, especially close to the luminal surface of the ventricular septum, but the ventricles of their age-matched normotensive control animals showed no signs of ANP immunoreactivity (Kinnunen et al., 1991). When transmural distribution of IR-ANP across the left ventricular wall was examined, the regional left ventricular IR-ANP concentration in WKY rats was 3.4- and 2.6-fold higher in the endocardium than in the outermost third and the midwall of the left ventricle, respectively (Ruskoaho et al., 1989a). The 1-year-old SHR evaluated in these studies had a 5.5-fold difference between endocardial and epicardial left ventricular IR-ANP concentrations and a 2.8-fold difference between the endocardium and midwall of the left ventricle (Ruskoaho et al., 1989a). The observations of transmural difference in IR-ANP concentration and its steeper gradient in the SHR with cardiac hypertrophy suggest that increased endocardial tension may explain the elevated left ventricular ANP synthesis.

Furthermore, in the WKY rat and SHR with diabetes induced by streptozotocin, left and right ventricular IR-ANP levels were elevated with a 2.5- to 3.2-fold increase in ANP mRNA levels (Matsubara et al., 1990a). Four weeks of insulin therapy restored the increased ANP mRNA levels to control levels. In this study, a significant correlation between ventricular end-diastolic pressures and ventricular IR-ANP levels was found (Matsubara et al., 1990a). These data indicate that ventricular ANP gene expression, as reflected by elevated ANP mRNA and IR-ANP levels and by immunohistochemistry, is markedly augmented in SHR at the stage of severe left ventricular hypertrophy and suggest that these genetically hypertensive rats are one of the best animal models for the study of the biosynthesis, storage, and secretion of ventricular ANP.

The participation of the ventricle in ANP expression in an animal model of spontaneous biventricular hypertrophy has also been described (Lee et al., 1988). A subpopulation of WKY rats that develop spontaneous biventricular hypertrophy in response to increased cardiac output with normal blood pressure have a marked accumulation of IR-ANP and ANP mRNA in both ventricles, with a 6-fold increase in ANP mRNA in the left ventricle. Right ventricular ANP mRNA levels equaled those found in the left ventricle and were strikingly greater than normal WKY rat right ventricular controls (Lee et al., 1988), showing that both the left and right ventricles can simultaneously respond to hypertrophy and increase ANP gene transcription.

Following chronic salt-induced hypertension, the cardiac ventricle in Dahl-S rats also contains substantially more IR-ANP and ANP mRNA compared with the Dahl-R strain (Dene and Rapp, 1987; Rapp and Dene, 1988). There was no strain difference between young (1.5 months of age) Dahl-S and Dahl-R rats, but in older animals (8.5 months of age), when the Dahl-S rats were markedly hypertensive, there was a 5- to 10-fold increase in both left ventricular IR-ANP and ANP mRNA levels in Dahl-S, but not Dahl-R, rats (Dene and Rapp, 1987). The levels of ANP seen in the ventricle of the hypertensive Dahl-S rat approached those seen in the newborn Dahl-S rat, suggesting that ventricular ANP increases in response to either hypertension or ventricular hypertrophy. The IR-ANP in ventricles was mainly present in the same precursor form ($proANP_{1-126}$) found in atria (Dene and Rapp, 1987).

The cardiomyopathic hamster, a genetic model of spontaneous cardiomyopathy and CHF, has been extensively studied (Currie et al., 1987; Ding et al., 1987; Cantin et al., 1988, 1990; Franch et al., 1988; Edwards et al., 1988a; Thibault et al., 1989c; Wong et al., 1992). As described earlier, in this model plasma IR-ANP concentrations are markedly increased and correlate with the degree of CHF. Ventricular IR-ANP (primarily as prohormone) and ANP mRNA increase substantially and correlate with the severity of cardiac insufficiency (Cantin et al., 1988; Edwards et al., 1988a; Thibault et al., 1989c; Wong et al., 1992). In animals with severe CHF, the ventricles have been reported to contain 13 to 100 times more ANP mRNA than control ventricles (Currie et al., 1987; Franch et al., 1988; Thibault et al., 1989c), and ventricular IR-ANP accounts for one-quarter of the total cardiac stores of ANP (Cantin et al., 1988; Franch et al., 1988). The ventricles of cardiomyopathic hamsters with CHF were estimated to contain up to 10-fold more ANP mRNA than the atria (Thibault et al., 1989c). The highest peptide levels were found in the inner half of the left ventricular wall (Cantin et al., 1988; Edwards et al., 1988a). The high-performance liquid chromatography pattern of IR-ANP was of the high molecular weight type both in the atria and ventricles of control and cardiomyopathic hamsters (Ding et al., 1987). Ventricular cardiocytes also showed an increase in the size of the Golgi complex, and secretory-like granules, identical with those in atrial cardiocytes, were present in greater numbers in about 20% of ventricular cardiocytes (Ding et al., 1987; Cantin et al., 1988, 1990). In hamsters with severe CHF, the prohormone is detectable in plasma, suggesting a defect in posttranslational processing (Ding et al., 1987; Thibault et al., 1989c).

The ANP gene expression in the ventricles is markedly increased in experimental myocardial infarction (Michel et al., 1988, 1990a; Bilder et al., 1989a; Drexler et al., 1989). In an experimental model of left ventricular infarction in rats, ANP mRNA increased significantly in the left (4-fold) and right (6-fold) ventricles, and treatment with an ACE inhibitor (perindopril) partially reversed this increase (Michel et al., 1988). Similarly, activation of right and left ventricular ANP mRNA levels by 1.9- and 3.8-fold, respectively, was observed 3 weeks after myocardial infarction in rats coupled with the increase in intracardiac pressures (Drexler et al., 1989). In myocardial infarctions, ventricular ANP mRNA positively correlated with right ventricular weight (Michel et al., 1990a), which is generally used as an index of the severity of heart failure in this experimental model. Ventricular IR-ANP levels increased only slightly with the progression of chronic heart failure produced by rapid ventricular pacing in dogs (Perrella et al., 1992). Furthermore, the ventricular concentrations of IR-ANP were low compared to atrial concentrations, and ANP mRNA was not detected in myocardium at any time (30 days) during CHF, probably because of the lack of ventricular hypertrophy in this canine model of CHF (Perrella et al., 1992).

Ventricular ANP expression has been described in pressure overload produced by banding of the abdominal aorta in rats (Day et al., 1987; Izumo et al., 1988; Mercadier et al., 1989). Constriction of the aorta for 1 week produced left ventricular hypertrophy, an increase in left ventricular wall thickness, and a 2- to 3-fold increase in

IR-ANP concentrations and ANP mRNA levels in the left but not right ventricle (Day et al., 1987). The total mass of ANP mRNA induced in the left ventricle was estimated to approach one-third of the mass of ANP mRNA in the atria. Combined aortic banding and dexamethasone treatment produced a 5-fold increase in left ventricular IR-ANP and ANP mRNA (Day et al., 1987). The accumulated mRNA in the left ventricle was identical with atrial mRNA, as judged by the transcriptional start site. Purification of ventricular extracts by high-performance liquid chromatography primarily demonstrated the presence of $proANP_{1-126}$ and small amounts of COOH-terminal peptide (Day et al., 1987).

Using the same aortic constriction model, Mercadier et al. (1989) found a biphasic accumulation of ANP mRNA in the left ventricle with a peak at day 4 averaging 20 times the control value before stable hypertension and hypertrophy was achieved, followed by a decrease until day 9. There then followed a second increase, which stabilized at approximately 10 times the control value seen during stable hypertension and hypertrophy. Gu et al. (1989) found ANP immunoreactivity and ANP mRNA, by in situ hybridization, in the left ventricle 2 days after coarctation, and their amounts increased in proportion to the intraventricular pressure and duration of coarctation. Their findings also suggest that the occurrence of ANP in overloaded ventricles may not be a transient, immediately reversible phenomenon, because, although the amount of ANP mRNA decreased with time, ANP-containing granules could still be found in the ventricular myocytes 77 days after the release of the coarctation (Gu et al., 1989, 1991). In rats with coarctations, no significant ANP mRNA accumulation was seen either 5 or 18 h after operation (Mercadier et al., 1989). The increase in left ventricular ANP synthesis was confirmed in all studies by immunocytochemical staining, demonstrating profuse granular staining throughout the left ventricular myocardium, strong staining being observed in the midwall and subendocardial areas (Day et al., 1987; Gu et al., 1989, 1991; Mercadier et al., 1989). Consistent with these studies, the ventricular concentrations of IR-ANP and ANP mRNA were 5.4 and 2.4 times higher in rats with aortic valve insufficiency than those in control rats (Morita et al., 1990), and murine myocardium showed a 35% increase in heart weight and a >20-fold increase in the ANP mRNA levels 7 days after aortic constriction (Rockman et al., 1991). In summary, these studies show that sustained elevations of blood pressure or volume load enhance ventricular ANP production.

Matsubara et al. (1990b) determined the changes in ventricular ANP gene expression in left ventricular hypertrophy and pressure overload induced by renovascular hypertension. Hypertension of 10 weeks' duration caused a 2-fold increase in the left ventricular hypertrophy, a significant increase in left ventricular end-diastolic pressure, and an 8-fold increase in left ventricular ANP mRNA levels in two-kidney, one-clip renovascular hypertensive rats, compared with levels in control rats (Matsubara et al., 1990b). In addition, the degree of left ventricular hypertrophy was directly correlated with left ventricular IR-ANP levels, supporting observations in other models of cardiac hypertrophy. Important, however, is the observation that the increased left ventricular ANP synthesis and left ventricular hypertrophy in hypertensive rats were reversed by enalapril treatment or removal of the clipped kidney but were not altered by hydralazine treatment. Furthermore, despite the absence of right ventricular hypertrophy, these treatments similarly increased right ventricular ANP synthesis (Matsubara et al., 1990b). These results suggest that factors other than hypertrophy play an important role in the regulation of the ventricular ANP response.

A hypertrophy-associated increase in ventricular ANP has been shown to occur in models producing an increase in pulmonary arterial pressure and right ventricular hypertrophy. Right ventricular pressure overload produced by hypoxic pulmonary hypertension leads to substantial induction of right ventricular IR-ANP and ANP mRNA (Stockmann et al., 1988; Winter et al., 1989; Raffestin et al., 1990). Rats exposed to a 10% oxygen environment for 3 weeks developed a 9-fold increase in right ventricular IR-ANP and a profound 160-fold increase in ANP mRNA levels (Stockmann et al., 1988). At the same time, a 4-fold increase in IR-ANP and 8-fold increase in ANP mRNA levels of left ventricles were found. Purification of ventricular tissue extracts by high-performance liquid chromatography primarily revealed the high molecular weight form prohormone (Stockmann et al., 1988). On the other hand, when Raffestin et al. (1990) used the same degree and duration of hypoxia, a 3.4- and a 2.9-fold increase in ANP mRNA concentration (relative to 18S ribosomal RNA) in the right and left ventricles was seen. Accumulation of ANP in the right ventricle followed the time course of development of ventricular hypertrophy induced by hypoxia (Stockmann et al., 1988; Winter et al., 1989). Interestingly, tissue concentrations of ANP rapidly decreased to control levels following return to normoxic conditions, preceding the complete regression of right ventricular hypertrophy (Stockmann et al., 1988); and despite enhanced ANP synthesis in the left ventricle, there was no evidence of left ventricular hypertrophy in the hypoxic rats (Raffestin et al., 1990). Thus, tissue hypoxia stimulates the induction of ANP synthesis, particularly in the right ventricle. Hypoxia-induced expression of ANP gene may require continuous stimulation and may have a direct effect on ANP gene expression. Accordingly, in another model of pulmonary hypertension produced by injection of monocrotaline, a time-dependent increase in the ventricular levels of IR-ANP (72-fold in the right ventricle) and ANP mRNA associated with right ventricular hypertrophy was observed (Oehlenschlager et al., 1989). Using this model,

Ceconi et al. (1989) reported that right ventricular IR-ANP concentration remained constant, whereas the content increased with hypertrophy, suggesting an increased rate of protein synthesis independent of the presence or absence of CHF.

ANP synthesis and storage are increased in ventricles of vasopressin-deficient Brattleboro rats (Lavigne et al., 1988; Ruskoaho et al., 1989b). In the ventricles, ANP mRNA and IR-ANP concentrations were about 2-fold higher in Brattleboro than in Long-Evans rats (Lavigne et al., 1988). Secretory-like granules with the size and density of atrial secretory granules were present in about 10% of ventricular cardiocytes dispersed throughout the thickness of the walls in the Brattleboro strain, but no IR-ANP-containing granules were seen in Long-Evans rats. In support of these results, normal Long-Evans had low levels of left ventricular ANP mRNA and barely detectable ANP mRNA in the right ventricle, whereas Brattleboro rats showed a 3-fold greater ANP mRNA in the left ventricle than age-matched Long-Evans controls (Ruskoaho et al., 1989b). Proportionally, the increase in the IR-ANP and ANP mRNA levels in the ventricles was greater than that in auricles. Interestingly, the ventricular weight to body weight ratio, the most commonly used index of the degree of hypertrophy, was similar in Long-Evans and Brattleboro rats (Ruskoaho et al., 1989b). The increased left ventricular levels of IR-ANP and augmentation of ANP mRNA levels in Brattleboro rats despite normal left ventricular weight show that increased cardiac gene expression may occur in the ventricles independently of hypertrophy.

Other experimental manipulations reported to influence ANP synthesis and storage include chemical sympathectomy and a diet high in sodium. Denervated ventricles (by 6-hydroxydopamine) showed a very high IR-ANP content 5 days after treatment (Albino-Teixeira et al., 1990). Furthermore, ANP mRNA was increased in both ventricles, particularly in the right ventricle, of animals receiving a high sodium diet when compared with animals maintained on diets low sodium (Lattion et al., 1988).

On the other hand, some investigators have reported that the amount of ventricular IR-ANP is reduced in the diseased ventricles. In one study of SHR, the ventricular levels of IR-ANP were reduced in the hearts of SHR compared with the normotensive controls; the decrease was greatest in 12-month-old SHR with the greatest ventricular hypertrophy (Ruskoaho and Leppäluoto, 1988a). The reason why these results differ from those previously mentioned is not clear, but variations in the ventricular IR-ANP concentrations in the WKY strain, the biventricular hypertrophy occasionally observed in the WKY rats (Lee et al., 1988), or the depletion of ventricular ANP in response to chronically increased pressure load may be responsible. Data supporting the last hypothesis was reported in a model of heart failure induced by pacing, in which both atrial and ventricular ANP storage were reduced in response to chronic stimulation of ANP synthesis and release during severe heart failure (Moe et al., 1991). The IR-ANP concentrations in the free walls of the left and right ventricles were higher than the corresponding concentrations in the controls in dogs with early heart failure. However, in dogs with severe failure, the IR-ANP concentrations were similar to controls, indicating reduced hormone content compared to early heart failure (Moe et al., 1991). Similarly, left ventricular ANP concentration and content of IR-ANP remained unchanged (Agnoletti et al., 1990a) or decreased in the heart failure group (Ceconi et al., 1989) induced by monocrotaline injection, suggesting a loss of ANP from the ventricle similar to that which occurs in the atria. Finally, hydralazine, a vasodilator, has been shown to decrease ANP mRNA levels, and this treatment did not significantly affect cardiac weight (Fukui et al., 1989). Thus, although the quantity of ANP mRNA as an indicator of ANP synthesis was not measured in all these studies, the observations of reduced or unchanged levels of IR-ANP with severe heart failure or hypertrophy suggest that either local synthetic mechanisms or storage or release of ANP is altered in ventricular tissue leading to depleted levels of ANP. Nevertheless, further studies are needed to clarify the importance of depletion of ventricular stores in response to chronic stimuli and whether the type and chronicity of hemodynamic changes produced in each model may be critical for specific alterations in ANP storage and synthesis.

2. Cardiac overload in humans. Ventricular ANP expression is also increased in humans with cardiac diseases. In a patient with DCM, the total ANP mRNA and IR-ANP levels in the ventricle were 40 to 80 times higher than in controls, and the total ANP mRNA content in the ventricle was about 30% of that present in the cardiomyopathic atria and 85% of that present in control atria (Saito et al., 1987b). The IR-ANP concentration of the diseased ventricle was 1 μg/g (and 0.02 μg/g in controls) and is comparable to that in the human fetal ventricle (Kikuchi et al., 1987; Saito et al., 1987b). Patients with DCM have IR-ANP granules within the right and left ventricular myocytes (Tsuchimochi et al., 1987, 1988; Edwards et al., 1988a; Yamada et al., 1988; Jougasaki et al., 1989; Takemura et al., 1989; Arbustini et al., 1990; Lee and Lee, 1990; Mochizuki et al., 1991). Interestingly, in the ventricles of patients with DCM, the IR-ANP contents were higher in the left ventricle than in the right ventricle (Tsuchimochi et al., 1988; Saito et al., 1989a; Takemura et al., 1989; Arbustini et al., 1990), and the left ventricular subendocardium contained more IR-ANP than the subepicardium (Edwards et al., 1988a; Tsuchimochi et al., 1988; Wharton et al., 1988a; Saito et al., 1989a; Arbustini et al., 1990; Nishikawa et al., 1990). This subendocardial location makes it feasible to identify ventricular ANP in tissue obtained

at endomyocardial biopsy. For example, ANP mRNA was abundant, as quantified by the polymerase chain reaction, in ventricular myocardium in endomyocardial biopsies from patients with DCM (Feldman et al., 1991). The total IR-ANP content in the dilated cardiomyopathic ventricle was 5% of that in the dilated cardiomyopathic atrium and total ANP mRNA content 30% of that in the dilated cardiomyopathic atria much the same as that in the normal atrium (Saito et al., 1989a). These results show that the ANP gene is reinduced in the ventricle of the patient with DCM and supports the concept that heart failure is a stimulus for the production of ANP by the ventricle.

Ventricular expression is not specific to DCM; increased ANP expression has been observed in the ventricles of patients with severe valvular heart diseases such as mitral stenosis, aortic regurgitation, and aortic stenosis (Tsuchimochi et al., 1988; Takemura et al., 1989; Mochizuki et al., 1991), ischemic heart disease and old myocardial infarction (Galipeau et al., 1988; Yamada et al., 1988; Saito et al., 1989a; Arbustini et al., 1990; Jougasaki et al., 1990; Takemura et al., 1990), ventricular dilation due to myocarditis (Takemura et al., 1989), myocarditis or myocardial infarction secondary to Kawasaki disease in children (Fujiwara et al., 1990), endocardial fibroelastosis (Nishikawa et al. 1990), restrictive cardiomyopathy (Edwards et al., 1990b), hypertrophic cardiomyopathy (Takemura et al., 1991), and hypertensive heart disease (Takemura et al., 1991). Thus, ventricular ANP is likely to be augmented in dilated hearts regardless of the cause of dilation. However, the amounts of ANP expressed in the ventricles of patients with DCM are considerably greater than amounts in other cardiac diseases (Tsuchimochi et al., 1988; Saito et al., 1989a; Takemura et al., 1989), and thus DCM could be identified as a cardiac disease that induces ventricular expression of ANP. In patients with a clinical diagnosis of restrictive heart disease, ventricular IR-ANP was detectable by ventricular endomyocardial biopsy (Edwards et al., 1990b), suggesting that its expression is not dependent on ventricular dilation. In patients with acute myocardial infarction, increased levels of ANP mRNA were noted in both ventricles, with the greatest increase in the infarcted ventricle (Galipeau et al., 1988). The difference of degree of ventricular expression may be explained by the differences of hemodynamics or histological variables rather than by disease (see below). Finally, myocardial synthesis does not appear to be altered in response to cardiac transplantation, because myocardial and conducting cells display ANP immunoreactivity at least up to 7 or 8 years after transplantation (Wharton et al., 1990). Thus, these observations suggest that the human ventricle can produce ANP in response to chronic pressure and volume overload, showing that this peptide may play a fundamental role in the maintenance and regulation of intravascular volume and blood pressure. Furthermore, the synthesis and storage of ventricular ANP, in contrast to atrial ANP, are both markedly increased in cardiac diseases, showing that they are different from the atrial processes.

3. Summary of the effect of pressure and volume overload on atrial natriuretic peptide gene expression. The combined measurements of ANP mRNA, atrial IR-ANP concentration, and plasma levels of IR-ANP show that increased intracardiac pressures by volume and pressure overload cause long-term stimulation of ANP synthesis and secretion of ANP into the circulation (table 2). In response to pressure and volume overload, ANP gene expression is activated in atrial and ventricular myocytes, which appears to depend largely on the activation of transcription of the ANP-coding gene. Up-regulation of the ANP gene in the atria results in a limited increase in ANP mRNA levels. On the other hand, pressure and volume overload is associated with qualitative changes in ventricular ANP mRNA levels, e.g., the reactivation of the ANP gene that is normally expressed in embryonic development. The induction of ANP gene expression in ventricles is very common, because it has been found in

TABLE 2
*Clinical disorders with changes in ANP synthesis, storage, and secretion**

Disease and reference	ANP synthesis (mRNA units/g)	ANP storage (μg/g)	ANP secretion (plasma IR-ANP)
Dilated cardiomyopathy			
Saito et al., 1987b	37 × ↑	50 × ↑	↑
Tsuchimochi et al., 1987	ND	↑ ihc	ND
Edwards et al., 1988a	ND	↑ ihc	ND
Tsuchimochi et al., 1988	ND	6 × ↑	ND
Yamada et al., 1988	ND	+ ihc	ND
Jougasaki et al., 1989	ND	↑ ihc	↑
Saito et al., 1989a	9 × ↑	10–77 × ↑	↑
Takemura et al., 1989	ND	↑ ihc	ND
Arbustini et al., 1990	ND	↑ ihc	ND
Edwards et al., 1990b	ND	+ ihc	↑
Feldman et al., 1991	↑	ND	ND
Myocardial infarct/aneurysm			
Galipeau et al., 1988	10–20 × ↑	ND	ND
Yamada et al., 1988	ND	+ ihc	ND
Saito et al., 1989a	9 × ↑	20–40 × ↑	↑
Arbustini et al., 1990	ND	↑ ihc	ND
Gu and McGrath, 1990	↑ is	↑ ihc	ND
Jougasaki et al., 1990	ND	↑ ihc	ND
Valvular heart disease			
Tsuchimochi et al., 1988	ND	3 × ↑	ND
Takemura et al., 1989	ND	↑ ihc	ND
Myocarditis			
Takemura et al., 1989	ND	↑ ihc	ND
Fujiwara et al., 1990	ND	↑ ihc	ND
Restrictive cardiomyopathy			
Edwards et al., 1990b	ND	+ ihc	↑
Hypertrophic cardiomyopathy			
Takemura et al., 1991	ND	↑ ihc	↑
Hypertensive hypertrophy			
Takemura et al., 1991	ND	↑ ihc	↑

* Abbreviations and symbols: ND, not determined; ihc, immunohistochemistry; ↑, increased; +, positive.

a wide variety of experimental species and in a wide variety of experimental models as well as in humans with cardiac pressure and volume overload. Of the known noncontractile protein genes, the reactivation of ANP gene expression may be one of the most well characterized. If this long-term stimulation is pronounced, atrial ANP concentration eventually decreases. The preliminary findings that ventricular ANP stores in severe heart failure, like those of atria, may be reduced in response to chronic stimulation of ANP synthesis and release remains to be confirmed.

4. Expression of brain natriuretic peptide in ventricular hypertrophy. Although BNP was first isolated from the brain, BNP is more abundant in cardiac ventriculocytes and is secreted from the heart into the circulation. Plasma IR-BNP levels are markedly increased in patients with CHF, chronic renal failure, hypertension, and myocardial infarction, and this increase is more prominent for BNP than ANP (Mukoyama et al., 1990a,b, 1991). The expression of the BNP gene in the normal human heart occurs with a rank order of right atrium > left atrium > right ventricle = left ventricle (Hosoda et al., 1991). In the atria, immunohistochemical studies revealed multipotential cells able to synthesize both natriuretic peptides (Hasegawa et al., 1991; Nakamura et al., 1991; Nakao et al., 1991; Pucci et al., 1992). BNP is mainly synthesized (Hosoda et al., 1991; Ogawa et al., 1991; Kohno et al., 1992b) and secreted from the ventricle in rats (Ogawa et al., 1991) and in humans (Mukoyama et al., 1991); in rats, the total amount of BNP mRNA in the ventricle represents about 75% of that in the whole heart (Hosoda et al., 1991; Ogawa et al., 1991).

Recently, the expression of the BNP gene has been shown to be elevated in certain experimental forms of cardiac hypertrophy and in human failing hearts. Preliminary results demonstrate that the total amounts of BNP mRNA in patients with DCM and hypertrophic cardiomyopathic hearts were increased to about 2- and 3-fold that of the normal heart, respectively (Hosoda et al., 1991; Mukoyama et al., 1991), and ventricular IR-BNP more than doubled in the failing heart (Mukoyama et al., 1991). This is consistent with the finding that the total content of BNP mRNA in the hypertrophied SHR-SP ventricle was 2-fold higher than that in age-matched WKY rat ventricles (Ogawa et al., 1991). In addition to ANP synthesis, ventricular IR-BNP production also was accelerated in DOCA/salt hypertensive rats (Yokota et al., 1990, 1991a) and in rats with nephrotic syndrome (Yokota et al., 1991b). Thus, the BNP synthesis and secretion are further enhanced in the ventricle of the hypertrophied heart, although the source of ANP in the heart shifts from the atrium to the ventricle. These results further suggest that the gene expression of ANP and BNP are differently regulated in the heart.

C. Glucocorticoids

Glucocorticoids have been shown to increase circulating ANP levels in the rat (Gardner et al., 1986b; Fullerton et al., 1991) and increase atrial (Gardner et al., 1986b; Day et al., 1987) and ventricular (Gardner et al., 1986b; Day et al., 1987; Fullerton et al., 1991) ANP mRNA levels in vivo. Garcia et al. (1985a) found that dexamethasone increased circulating ANP levels in adrenalectomized rats if given together with DOCA, but no effect was noted in the absence of the mineralocorticoid. Tonolo et al. (1988) reported that 2 μg of dexamethasone reduced plasma IR-ANP concentration in rats. Dexamethasone treatment (4 days) increased atrial IR-ANP content and release of IR-ANP from minced atrial tissue (Lachance et al., 1986). Administration of dexamethasone for 48 h to either intact or adrenalectomized rats increased plasma IR-ANP levels by about 2-fold, atrial and ventricular ANP mRNA levels by 1.5- to 2.5-fold in adrenalectomized rats, and atrial ANP mRNA levels 2-fold in water-deprived rats (Gardner et al., 1986b). Similarly, Day et al. (1987) reported 2.4- to 3.6-fold increases in left ventricular IR-ANP concentration and ANP mRNA content in response to 1 to 2 days of dexamethasone treatment in rats, whereas it decreased plasma and atrial concentrations of IR-ANP. Combined aortic banding and dexamethasone treatment produced a 5-fold increase in left ventricular ANP and ANP mRNA. These interventions had no effect on ANP expression in the right ventricle (Day et al., 1987). Left ventricular IR-ANP and ANP mRNA concentrations as well as plasma IR-ANP levels also increased with dexamethasone treatment in adrenalectomized rats, whereas atrial levels were reported to remain unchanged (Fullerton et al., 1991).

In vitro studies show that glucocorticoids act directly on myocardial cells to increase ANP mRNA levels. Matsubara and colleagues reported that dexamethasone increased the release of IR-ANP from primary cultures of atrial (Matsubara et al., 1987a) as well as ventricular cells (Matsubara et al., 1987b); the latter appeared to be more sensitive to the steroid, and was confirmed later by Argentin et al. (1991). Administration of dexamethasone increased cellular ANP mRNA in neonatal rat atrial (Gardner et al., 1988; Shields et al., 1988; Argentin et al., 1991) and ventricular cells (Shields et al., 1988; Argentin et al., 1991). The increase in media IR-ANP occurs in a dose-dependent fashion within 2 h of exposure to dexamethasone (Gardner et al., 1988). Also, increase in ANP mRNA levels is rapid; when ventricular cells were exposed to 10^{-7} M dexamethasone for varying amounts of time, maximal induction of ANP gene expression was observed after only 6 h of treatment (Argentin et al., 1991). The effect of dexamethasone was maximal between 10^{-8} and 10^{-7} M dexamethasone, which is compatible with the known K_d of dexamethasone for the glucocorticoid receptor (Gardner et al., 1988; Argentin et al., 1991). Treatment of atrial enriched myocardial cells with dexamethasone also increased the amount of ANP mRNA visualized by in situ hybridization (Gardner et al., 1988). Dexamethasone caused modest, but statisti-

cally insignificant, increases in the $t_{1/2}$ (18 to 30 h) of the ANP mRNA that could serve to enhance the transcriptional response to the hormone (Gardner et al., 1988). The increase in ANP mRNA appears to be relatively specific for glucocorticoids; DOCA, progesterone, and estradiol do not affect ANP mRNA levels in atrial cells (Gardner et al., 1988). RU 34846, a specific glucocorticoid antagonist, inhibited both the increase in cellular ANP mRNA and the increase in secreted peptide seen after addition of dexamethasone (Gardner et al., 1988). Yet, Matsubara et al. (1987a,b) reported that testosterone was almost equipotent to dexamethasone in increasing IR-ANP in atrial and ventricular cells, and corticosterone, DOCA, aldosterone, and progesterone also had a statistically significant stimulatory effect on the concentration of IR-ANP in atrial cells. Human ANP gene with SV40 enhancer has even been reported to be negatively regulated by dexamethasone (Iwai et al., 1987), and no effect was observed with glucocorticoids in adult rat ventricular cells (Claycomb, 1988).

The increase in the biosynthesis of ANP in response to glucocorticoids is consistent with the presence of DNA sequences in the second intron of the human (Greenberg et al., 1984; Seidman et al., 1984a) and rat (Argentin et al., 1985) gene that bear some homology to the glucocorticoid receptor-binding consensus sequence; whereas the mouse gene lacks this binding site (Seidman et al., 1984a). Argentin and colleagues (1991) recently reported that purified glucocorticoid receptors bind to an upstream element required for hormone responsiveness in the rat gene. Expression of reporter genes is increased by glucocorticoids when these are transcribed under the direction of ANP rather than other regulatory sequences. To the extent that these effects are primary, one would expect this increased gene expression to be mediated by appropriate hormone receptors that interact with specific DNA sequences, as has been demonstrated in other systems. Thus, the data support the primary role of glucocorticoids on ANP gene transcription.

D. Thyroid Hormones

Thyroid hormones have a number of effects on cardiovascular and renal function and can also regulate ANP gene expression. Circulating IR-ANP levels are elevated in experimental animals (Kohno et al., 1986; Ladenson et al., 1987; Wong et al., 1989) and patients with hyperthyroidism (Kohno et al., 1987b; Ladenson et al., 1987). IR-ANP content and concentration in the atria are lower in hyperthyroid rats than in hypothyroid rats, and administration of large doses of T_4 to intact rats increases plasma IR-ANP levels above their control levels (Kohno et al., 1986; Wong et al., 1989). Administration of T_4 to thyroidectomized rats that were dehydrated to reduce baseline ANP gene expression increased pooled atrial ANP mRNA levels by about 3-fold and plasma IR-ANP concentration by 2-fold, whereas no changes in ANP were observed in water-repleted rats (Gardner et al., 1987b). Ventricular ANP mRNA levels also increased by about 60% in response to T_4 treatment in these animals. Ladenson et al. (1988) showed that both the left (272%) and right (176%) atrial ANP mRNA contents but not concentrations in hyperthyroid rats were increased with a decrease (24%) in left atrial IR-ANP content. Consistent with that study, Fullerton et al. (1990) showed increased left and right atrial ANP mRNA levels (pooled samples) in T_4-treated normally hydrated rats and reduced levels in propylthiouracil-treated animals. T_4 treatment decreased right atrial IR-ANP concentration, whereas propylthiouracil markedly increased left ventricular IR-ANP concentration (Fullerton et al., 1990). In support of these studies, ANP release from the atria of hypothyroid rats was significantly decreased (Wong et al., 1989).

Thyroid hormones also increase the expression of ANP in vitro. Treatment of neonatal cardiocytes (atrial plus ventricular) with triiodothyronine at concentrations ranging from 10^{-10} to 10^{-8} M for 24 h resulted in up to a 2-fold increase in ANP mRNA levels and release of IR-ANP into the medium (Gardner et al., 1987b). There was also a significant increase in media IR-ANP as early as 2 h after addition of triiodothyronine. In other in vitro studies, neonatal atrial or ventricular ANP synthesis and release by cultured cardiocytes were also enhanced by thyroid hormone exposure (Argentin et al., 1987; Matsubara et al. 1987a,b; Rundle et al. 1990), although no effect was observed in adult ventricular cell cultures (Claycomb, 1988). Argentin et al. (1987) reported a 2-fold increase in ANP mRNA levels in atrial cells and a 4-fold increase in ventricular cells exposed to thyroid hormone; the effect on ANP mRNA level was apparent at 12 h and maximal at 48 h after addition of triiodothyronine. The effect of T_4 on atrial ANP gene expression is induced after it is converted to triiodothyronine, probably by type I T_4 deionidase, because propylthiouracil, but not methimazole, inhibited the effects of T_4 (Mori et al., 1990).

Although these studies do not define the exact mechanisms of action of thyroid hormone on ANP gene expression, direct induction and nonspecific hypertrophic action of thyroid hormone may both contribute to the observed increase in ANP mRNA levels. Furthermore, the increase in ANP synthesis induced by thyroid hormones may be a secondary effect of the hemodynamic changes, as suggested by the findings that in vivo ANP mRNA levels changed with associated changes in stored ANP levels similarly to that observed in volume and pressure models. Nevertheless, the increased myocardial ANP production may contribute to the hemodynamic and renal manifestations of thyroid hormone excess and deficiency.

E. Other Humoral and Growth Factors

1. Endothelin. ET-1, a naturally occurring peptide derived from endothelial cells, is a potent constrictor of

vascular smooth muscle (for reviews, see Yanagisawa and Masaki, 1989; Masaki et al., 1991; Rubanyi and Botelho, 1991). When cultured neonatal ventricular cells were used, ET-1 was shown to increase ANP mRNA in cardiocytes (Fukuda et al. 1989; Shubeita et al., 1990; Gardner et al., 1991). ANP mRNA levels in ET-1-treated atrial cells increase about 1.4-fold over nontreated cells as early as 3 h after administration (Fukuda et al., 1989). Maximal increases in ANP mRNA levels (5-fold) with 10 nM ET-1 occurred by 48 h (Shubeita et al., 1990). The induction of ANP mRNA was associated with an increase in the transcription of the ANP gene, as shown by a 6.9-fold induction of an ANP-promoter construct carrying the coding portion of the firefly luciferase reporter gene transfected into neonatal rat ventricular cells. Shubeita et al. (1990) also showed that ET-1 induced myocyte cell hypertrophy and activated immediate early (*c-fos*, *egr-1*) and contractile protein (myosin light chain-2) gene expression. ET-dependent stimulation of IR-ANP release was not limited by cell density of the culture (Gardner et al., 1991). Because ET-1 is released from endothelial cells that lie immediately adjacent to the myocytes within the intact myocardium, the activation of ANP gene expression by ET-1 may represent a potentially important paracrine mechanism for the regulation of ANP gene expression. Furthermore, it is important to study whether endothelial cells might represent the primary sensor for mechanical stretch through the augmented release of ET-1, thereby providing a potential mechanism for the transduction of a mechanical stimulus into the activation of ANP gene in myocardial cells, because ET has been shown to enhance atrial stretch-induced ANP release (Mäntymaa et al., 1990; Gardner et al., 1991; Schiebinger and Greening, 1992).

2. *Catecholamines.* α_1-Adrenergic agonists can activate ANP gene expression in neonatal rat ventricular myocardial cells (Knowlton et al., 1991; Sei et al., 1991; Shubeita et al., 1992). Treatment with phenylephrine (100 μM) resulted in a time-dependent increase in ANP mRNA levels, with the increase detectable as early as 6 h and reaching maximum levels (10- to 15-fold) between 24 and 48 h. The removal of phenylephrine resulted in a rapid decline in ANP gene expression (Sei et al., 1991), suggesting that the sustained elevation of some intracellular messenger is required for the adrenergic response. Utilizing an RNAase protection assay, Knowlton et al. (1991) observed an increase in ANP mRNA levels of >15-fold. Phenylephrine treatment also resulted in a 5-fold increase in the expression of ANP-promoter/luciferase-reporter gene showing that α-agonist treatment leads to increased transcription of the ANP gene (Shubeita et al., 1992). The activation of ANP synthesis appeared to be mediated by α_1-receptors, because treatment with prazosin significantly blunted the stimulatory effect of noradrenaline on ANP release from ventricular myocytes (Knowlton et al., 1991).

3. *Growth factors.* Peptide growth factors have also been shown to modulate ANP gene expression. In cultured cardiac muscle cells, peptide growth factors regulate a number of genes, including α- and β-myosin heavy chains and cardiac and skeletal α-actin, provoking a generalized "fetal" phenotype similar to transitions elicited during adaptation to chronic pressure overload hypertrophy (Schneider and Parker, 1990). When cardiac myocytes isolated from the neonatal rat ventricles were tested by RNA blot hybridization, acidic and basic fibroblast growth factors, as well as transforming growth factor type β1, all augmented ANP mRNA levels 2- to 5-fold (Parker et al., 1990). These results indicate that peptide growth factors modulate cardiac expression of an ensemble of genes, encoding not only contractile proteins but also the secreted peptide, ANP. Neither the mechanisms nor the *cis*-acting sequences mediating activation of ANP gene expression in response to peptide growth factors are known.

4. *Prostaglandins and other products of arachidonic acid metabolism.* PGs, including $PGF_{2\alpha}$ and PGE_2, are able to directly increase ANP synthesis and secretion in cultured neonatal atrial and ventricular cells (Gardner and Schulz, 1990; Kovacic-Milivojevic et al., 1991). Treatment with $PGF_{2\alpha}$ and PGE_2 resulted in a stimulation of ANP mRNA levels at 24 h over a dose range (10^{-7} to 10^{-5} M) that was similar to that found for secretion of the peptide, whereas prostacyclin had no effect on ANP secretion under these conditions. This effect appeared to be independent of cell density in the cultures (Kovacic-Milivojevic et al., 1991). Transient expression analysis of atrial cells transfected with 2500 bp of human ANP 5′-flanking sequences linked to a CAT reporter demonstrated that $PGF_{2\alpha}$ (10^{-5} M) increased promoter activity approximately 2-fold relative to the control (Gardner and Schulz, 1990), suggesting that the increase in ANP mRNA levels was derived, at least in part, from augmented transcription of the ANP gene. Furthermore, high concentrations of indomethacin, meclophenamate, and naproxene resulted in significant reductions in IR-ANP release at 24 h, implicating a role for PGs in maintaining basal ANP biosynthesis. When several pharmacological inhibitors of the second-messenger systems were used, the stimulatory effect of PGs appeared to be mediated by a calmodulin-dependent step (Gardner and Schulz, 1990; Kovacic-Milivojevic et al., 1991). Furthermore, several inhibitors of lipoxygenase activity, including nordihydroguarietic acid, baclein, and caffeic acid, proved effective at high concentrations in suppressing IR-ANP release at 24 h after their addition (Kovacic-Milivojevic et al., 1991). Although these agents are not specific for the lipoxygenase pathway, leukotriene C_4 treatment of atrial myocytes for 24 h also caused a modest increase in IR-ANP release, suggesting that other products of arachidonic acid metabolism, in addition to $PGF_{2\alpha}$ and PGE_2, may regulate ANP biosynthesis and

secretion. Taken together, results of these studies suggest that the eicosanoids play a significant role in regulating ANP synthesis, but the relative contribution of various arachidonate metabolism products remains to be established.

5. Other substances. Testosterone, selenium, transferrin, and insulin are also important for the maintenance of culture ANP content (Glembotski et al., 1987). No significant effect on ANP mRNA levels in adult ventricular cell culture was reported after treatment with isoproterenol, ANG II, AVP, enkephalinamide, and ANP_{99-126} (Claycomb, 1988).

F. Mechanisms of Induction of Atrial Natriuretic Peptide-coding Gene in Cardiac Myocytes

1. Stimuli for atrial natriuretic peptide gene expression. Although the alterations in the expression of the ANP gene have been well characterized, relatively little is known about the precise stimulus for ANP gene expression in the atrial and ventricular myocytes in response to pressure and volume overload. Yet, the data summarized above and in table 1 show that the activation of ANP gene expression is a complex and multifactorial phenomenon. The final common pathway may be mediated by a direct myocyte stretch and/or through humoral (e.g., glucocorticoids, catecholamines) and trophic factors (growth factors, ET). As we have seen, ventricular hypertrophy, induced by pressure and volume overload of the heart both in patients with heart failure and hypertension and in numerous experimental animal models, is characterized by augmented IR-ANP and ANP mRNA levels, supporting the view that ANP gene expression is a direct response to cell hypertrophy. Furthermore, the site and extent of ANP synthesis correlates well with the site and extent of hypertrophy both in vitro and in vivo. However, whether the left ventricular ANP gene expression is only a biochemical marker of myocardial hypertrophy or whether ANP gene expression and left ventricular hypertrophy are both independent consequences of mechanical overload which happen to increase in a parallel manner is not yet clear. Thus, further studies are needed to clarify the precise signals of the augmented expression of the ANP gene in the hypertrophied ventricle and the relationship between the ANP gene expression and ventricular hypertrophy.

There is substantial biochemical and morphological evidence that hypertrophy induces the myocyte to express fetal characteristics (for reviews, see Swynghedauw, 1986; Nadal-Ginard and Mahdavi, 1989; Katz, 1990). For example, cardiac overload in the adult rat results in the redistribution of ventricular myosin to the fetal β-isoform. As discussed earlier, during embryonic development, the ANP gene is expressed in both the atrium and the ventricle, and fetal mammalian ventricular cells contain electron-dense, membrane-bound granules that resemble atrial granules. Soon after birth, the expression of ANP is down-regulated in the ventricle with disappearance of the granules, and the atrium is the primary site of ANP synthesis within the mature myocardium. After induction of ventricular hypertrophy, there is reexpression of ANP in ventricular cells, and ultrastructural studies show similarities between fetal and hypertrophied adult ventricular myocytes. Therefore, reexpression of ventricular ANP synthesis in response to cardiac overload and with development of ventricular hypertrophy may represent the activation of an embryonic program of gene expression in hypertrophied ventricular muscle cells. Ventricular hypertrophy might, therefore, cause a generalized induction of developmentally regulated genes, and ANP gene expression may thus be a useful biochemical marker for identifying reexpression of embryonic genes by adult ventricular cells.

The view that myocyte stretch itself stimulates ANP gene expression is conceptually attractive because atrial stretch appears to be the primary stimulus for ANP release from the atria (Lang et al., 1985). Evidence supporting this hypothesis includes data showing the different patterns of the ANP distribution in the right ventricle and left ventricle during development and in pathological adult hearts. In fetal ventricles, ANP contents are higher in the right ventricle than in the left ventricle (Tsuchimochi et al., 1988), but in the patients with DCM, ANP tissue levels are higher in the left ventricular subendocardium than in the right ventricular subendocardium (Edwards et al. 1988a; Tsuchimochi et al., 1988). Because the right ventricle pumps against a higher pressure than the left ventricle in the fetal period, and the ventricular pressure is greater in diseased left than right ventricle, increased intramural tension may represent the stimulating factor necessary for the synthesis of ANP.

Further support for the view that myocyte stretch itself stimulates ANP gene expression includes data from both experimental animals and humans showing that an increase in after- or preload (systolic or diastolic dysfunction) is associated with the induction of the ANP gene. Several studies have shown that left ventricular ANP expression, measured by the presence of ANP-positive myocytes by immunohistochemistry or by measuring IR-ANP and ANP mRNA levels, correlates positively with pulmonary capillary wedge pressure, left ventricular end-diastolic pressure, peak systolic or end-diastolic wall stress, and with left ventricular end-systolic or end-diastolic volume index (Drexler et al., 1989; Gu et al., 1989; Mercadier et al., 1989; Saito et al., 1989a; Takemura et al., 1989; Arbustini et al., 1990; Edwards et al., 1990b; Matsubara et al., 1990b; Raffestin et al., 1990). Furthermore, the concentrations of ANP in and around a left ventricular aneurysm or infarct are high and decrease in myocytes located more distant from the left ventricular aneurysm or infarct (Saito et al., 1989a; Gu

and McGrath, 1990; Takemura et al., 1990; Gu et al., 1991). This further supports the concept that regional mechanical wall stretch is closely associated with the ventricular ANP gene expression, because the formation of granulation or fibrosis following myocardial infarction causes a reduction in the absolute transmural pressure, and the cells around lesions are subject to a more vigorous stretching force than other myocytes in the same chamber. The observation that intramural tension is greatest within the subendocardial region of the ventricle with the greatest ANP immunoreactivity also agrees with the concept of stretch-induced ANP gene expression. Thus, increased cardiac filling pressure resulting in increased wall stress or distension may represent a common hemodynamic stimulus for the expression of ANP within the ventricular myocardium.

Preliminary in vitro observations support the concept that direct mechanical stretch activates ANP gene expression. Tokola et al. (1991b) used the hypoosmolality-induced swelling of the cultured neonatal rat myocytes as an experimental model to examine whether cellular stretch affects ANP gene expression. Lowering the osmolality moderately from 289 to 245 mOsm/kg H_2O for 24 h caused a 26% increase in ANP mRNA levels in atrial cells (related to 18S ribosomal RNA), comparable to the in vivo studies (Tokola et al., 1991b). Thus, the osmotic stretch of atrial plasma membrane appears to stimulate ANP gene expression as well as IR-ANP release (Greenwald et al., 1989) under these experimental conditions. Preliminary findings from isolated perfused rat hearts show that myocyte stretching induced by increasing the volume of a left ventricular balloon produced a rapid, 2.1-fold increase in left ventricular ANP mRNA levels determined by Northern blot analysis in paced hearts after 30 min of constant stretch (Mäntymaa et al., 1991). A similar, but less marked (29%) increase in the atrial ANP mRNA level was observed after 4 h of constant right atrial stretch. Furthermore, when primary neonatal rat cardiocytes were cultured on flexible-bottomed collagen-coated culture dishes in a serum-free medium, mechanical cyclic or static loading (10%, from 1 to 48 h) of atrial myocytes stimulated ANP gene expression (Tokola et al., 1991a). Similarly, static stretch (20%, from 12 to 48 h) of the neonatal myocytes grown on a stretchable substrate caused an accumulation of ANP mRNA (Sadoshima et al., 1992). These reports suggest that myocardial stretch without the participation of humoral factors directly increases the ANP gene expression.

Despite the evidence summarized above that cardiac filling pressure with or without hypertrophy may stimulate ventricular ANP synthesis, alternative explanations should be considered, including the possible role of humoral factors and growth factors. Of particular importance may be the nonmyocardial cells present in the myocardium, because they appear to produce factors that can influence ANP secretion and ANP gene expression. In addition, intracellular hypoxia and metabolic changes produced by myocyte stretch may represent the underlying stimulus for ANP expression in the ventricle. Hypoxia followed by normoxia caused right ventricular IR-ANP to decrease to control levels within 3 days, despite persistent right ventricular hypertrophy (Stockmann et al., 1988). Plasma renin activity also increases simultaneously with ANP mRNA after aortic coarctation, suggesting that activation of the renin-ANG-aldosterone system might be involved in the regulation of the ANP gene expression (Mercadier et al., 1989). Furthermore, ascending aortic constriction increased the level of ANP mRNA in the adult rats but failed to cause a further significant increase in ANP gene expression in the old animals (Takahashi et al., 1992). All these data show the complexity of gene reprograming at the onset of cardiac overload and demonstrate that multiple factors are probably involved to mutually regulate the activation of ANP gene expression.

In summary, in pressure and volume overload, a common dominator is an increase in myocyte stress or distension, probably the major factor activating ANP gene expression both in atrial and ventricular cells. In addition, several autocrine and paracrine mechanisms appear to coexist for initiating or mediating cardiac overload-induced ANP gene expression. Why these stimuli lead, in the case of atrial cardiocytes, to an actual decrease in IR-ANP content in response to overload, whereas the reverse occurs in ventricular cardiocytes, remains to be determined. It may be a reflection of the fact that the secretory processes in atria and ventricles are different. The reactivation of the ANP gene in ventricles in pressure and volume overload states shows that the reinduction of ventricular ANP represents a fundamental adaptive response of the heart to increased load, in addition to myocardial cell hypertrophy and Frank-Starling mechanisms. Thus, cardiac overload with chronic ventricular myocyte stretch stimulates the ventricular cardiocytes to synthesize and secrete ANP in an attempt to compensate for the increased load. It is also possible that the increased ventricular ANP level has some local importance in the hypertrophied ventricle. It is interesting in this regard that the ANP precursor gene and the epidermal growth factor precursor gene have areas of homology (Hayashida and Miyata, 1985) and that ANP has growth inhibitory effects at least on vascular smooth muscle cells (Abell et al., 1989a; Itoh et al., 1990). Finally, the existence of mechanical and hormonal stimuli that activate cardiac muscle cell hypertrophy has been appreciated for many years. Therefore, ANP studies may help in understanding the stimuli and signaling mechanisms that lead to the transcriptional activation of other protein and embryonic target genes during myocardial cell hypertrophy.

2. Molecular basis for activation of atrial natriuretic

peptide gene expression. A major challenge is to clarify the molecular basis for activation of ANP gene expression in response to pressure and volume overload. Obviously, mechanical stretch as well as autocrine and paracrine stimuli must generate intracellular signals which ultimately reach the nucleus and activate the transcription of the ANP gene. An analysis of the 5′-flanking sequences of the rat and human ANP genes has indicated the presence of consensus sequences for several well-characterized transcriptional regulatory proteins (see section II.B). In the region from −496 to −489 in the rat promoter (Seidman et al., 1988; Rosenzweig et al., 1991) and region −241 to −235 in the human gene (Wu et al., 1991; Kovacic-Milivojevic and Gardner, 1992), there is consensus sequence for the AP-1 (fos/jun) binding, and in vitro binding of the c-*fos*/c-*jun* heterodimer to ANP sequences has been reported by Rosenzweig et al. (1991), suggesting that the heterodimer may play a role in the regulation of ANP gene transcription. Furthermore, using transient transfection analysis in neonatal rat cardiocytes, Kovacic-Milivojevic and Gardner (1992) showed that overexpression of c-*jun* results in a dose-dependent induction of the human ANP gene promoter. Overexpression of c-*fos* had a biphasic effect on human ANP gene promoter activity. At low levels, in concert with c-*jun*, c-*fos* stimulated, whereas at higher levels, it inhibited transcription from the human ANP gene promoter (Kovacic-Milivojevic and Gardner, 1992). These studies suggest that the components of the AP-1 complex either alone or in combination could influence ANP gene regulation.

Among the various stimuli that activate fos/jun heterodimer formation and, thus, binding to and regulation of certain AP-1-containing genes are those that stimulate PKC (Hunter, 1991). The findings that stimulation of PKC by phorbol esters increases ANP secretion from atrial (Ruskoaho et al., 1985) and ventricular cells (Kinnunen et al., 1991) and that the expression of the ANP gene is increased in cardiac myocytes treated with phorbol esters (Kovacic-Milivojevic and Gardner, 1992; Shubeita et al., 1992) or phorbol esters in combination with calcium ionophore A 23187 (LaPointe et al., 1990) suggest that PKC-regulated transcriptional factors may be involved in mediating the ANP activation. In support of this concept, ET and α_1-adrenergic agonists, which are among the most effective stimulators of ANP gene expression in vitro, have been shown to activate phosphoinositide hydrolysis in cardiac myocytes (Brown et al., 1985; Shubeita et al., 1990; Sei et al., 1991) leading to IP_3-mediated mobilization of intracellular calcium (Berridge, 1987) and DAG-mediated activation of PKC (Nishizuka, 1986). Phosphoinositide turnover also appeared to be the likely mediator of PG-stimulated ANP secretion in cultured atrial myocytes (Gardner and Schulz, 1990; Kovacic-Milivojevic et al., 1991), and a PKC inhibitor, H-7, caused an approximate 75% reduction in phenylephrine-stimulated ANP expression in neonatal rat ventricular cells (Sei et al., 1991). Furthermore, myocardial stretch modifies the intracellular content of several signaling compounds, including inositol phosphates (von Harsdorf et al., 1989). Thus, stretch-mediated increases in phosphoinositol hydrolysis and the resulting activation of PKC could contribute to induction of ANP gene expression. Consistent with the activation of the ANP gene by PKC, cotransfection of vectors that direct the expression of a constitutively active PKC with ANP/luciferase fusion genes leads to a severalfold increase in luciferase reporter activity (Shubeita et al., 1992). Catalytically inactive PKC constructs had no effect on the cardiac promoter constructs (Shubeita et al., 1992). Yet, although results of these studies suggest that PKC activation can lead to the activation of ANP gene, direct evidence for a role of PKC in the activation of the ANP gene in response to mechanical stretch and other stimuli is still needed.

In addition to PKC-dependent pathways, other known intracellular signal pathways and kinases, such as cAMP-protein kinase A, tyrosine kinases, and Ca^{2+}/calmodulin-dependent kinases, may have a significant role in the activation of the ANP-coding gene. Preliminary experiments have suggested (although actual data was not shown) that in primary cardiac myocytes cAMP does not stimulate ANP gene expression (Knowlton et al., 1991; Sei et al., 1991). Yet, two cAMP-dependent agonists, 8-bromo-cAMP and forskolin, suppressed IR-ANP accumulation into the incubation medium at 24 h, and isobutylmethylxanthine, a phosphodiesterase inhibitor, and forskolin resulted in partial reductions of the $PGF_{2\alpha}$ effect (Gardner and Schulz, 1990), suggesting decreased ANP production in response to cAMP-protein kinase A. In contrast, dibutyryl-cAMP increased ANP mRNA levels in cultured human fetal ventricular cells (Claycomb, 1988). The evidence that tyrosine kinase might be involved in the regulation of ANP gene expression is based on the stimulatory effect of peptide growth factors on ANP mRNA levels in ventricular cardiocytes. At least four structural classes of growth factor receptor have been identified, all of which activate tyrosine kinases (Schneider and Parker, 1990).

Because changes in intracellular [Ca^{2+}] accompany variations in the contractile state of the myocardium, the alterations of intracellular Ca^{2+} levels could represent an appropriate stimulus for the activation of ANP gene expression in response to pressure and volume overload. In cell culture models, Ca^{2+} and contraction can activate several features of myocardial cell hypertrophy, including the acceleration of RNA and protein synthesis and activation of c-*fos* and c-*jun* (Chien et al., 1991; Morgan and Baker, 1991). In neonatal atrial and ventricular cells, a dose-dependent increase between cytoplasmic ANP mRNA concentration and extracellular [Ca^{2+}] over a range of 0 to 2.0 mM was found by LaPointe et al. (1990).

When cardiocytes were treated for 24 h with the calcium channel blockers nifedipine or verapamil, both synthesis and secretion decreased to 25 to 40% of control values; when the extracellular [Ca^{2+}] of the media of cells treated with verapamil was increased, the secretion of ANP was partially restored. A calcium ionophore that increases the intracellular calcium levels increased IR-ANP release into the medium and in combination with phorbol ester enhanced ANP gene expression (LaPointe et al., 1990). Addition of increasing concentrations of KCl for 24 h into the culture medium led to a dose-dependent increase in IR-ANP release (Gardner and Schulz, 1990). Furthermore, when calcium influx was stimulated with Bay K8644, there was no increase in phosphoinositol hydrolysis, but a significant increase in ANP gene expression, estimated by ANP mRNA levels and by IR-ANP accumulation into the incubation medium of primary neonatal rat ventricular cell cultures, was seen (Sei et al., 1991). Nifedipine, a calcium channel antagonist, inhibited Bay K8644- and phenylephrine-dependent increases in ANP mRNA levels, and W-7, a Ca^{2+}/calmodulin inhibitor, completely blocked the effects of both phenylephrine and Bay K8644 on ANP production (Sei et al., 1991). Treatment of atrial cardiocytes with the calmodulin antagonist calmidazolium for 24 h reduced basal secretion rate and eliminated the $PGF_{2\alpha}$-stimulated ANP release, suggesting that ANP release by PGs involves a calmodulin-dependent step (Kovacic-Milivojevic et al., 1991). Thus, it appears that Ca^{2+} and calmodulin augment ANP gene expression through mechanisms not requiring PKC. Interestingly, in several cases, it has been shown that the calcium response elements are indistinguishable from cAMP response elements and between −602 and −596 bp in the rat ANP promoter is a sequence that is similar to the cAMP-responsive element consensus sequence (Seidman et al., 1988; Rosenzweig et al., 1991). Thus, there is a possibility that the cAMP-responsive element sequence in the ANP promoter may serve as a calcium-response element, and a critical evaluation of calcium and other ionic messengers in the activation of ANP gene is required.

Recently, a negative calcium-responsive element, originally found in the human parathyroid gene, was detected in the upstream regions (between −2921 and −2907 bp) of the rat ANP gene (Okazaki et al., 1992). Expression of the ANP gene was reported to be negatively regulated by extracellular Ca^{2+} by using an in vivo rat infusion system; the level of ANP mRNA from the heart of the calcium-infused rats was markedly lower compared with that from the rats without calcium (Okazaki et al., 1992). Transfection of the cultured cells further suggested that the element conferred negative regulation by extracellular [Ca^{2+}] on the reported gene. The role of this negative calcium-responsive element in the regulation of ANP transcription remains to be clarified.

Pressure overload also generates an intranuclear signal in the form of increased expression of protooncogenes (Mulvagh et al., 1988; Izumo et al., 1988; Komuro et al., 1988, 1990; see Chien et al., 1991). The earliest effect of mechanical stretching on gene expression in rat myocardial cells is the rapid induction (within 15 to 30 min) of a program of immediate early gene expression, which includes the c-*fos* and c-*jun* genes (Komuro et al., 1988; 1990). In vitro binding of the c-*fos*/c-*jun* heterodimer to ANP sequences (see above) and increased expression of c-*fos* and other protooncogenes (Izumo et al., 1988; Komuro et al., 1988) as well as ANP-coding gene in pressure and volume overload hypertrophy suggest that the c-*fos*/c-*jun* heterodimer may play a role in regulating ANP gene expression during states of increased protooncogene expression, such as ventricular hypertrophy. The region may be of considerable importance in regulating ANP response to diverse stimuli, because, as discussed before, the ANP gene is sensitive to phorbol esters and other PKC-activating agents.

At present, however, there is no direct evidence that immediate early genes are either necessary or sufficient to activate ANP gene expression. There are low (or undetectable) amounts of these protooncogenes in normal cardiac tissue (Izumo et al., 1988; Komuro et al., 1988), suggesting that the AP-1-binding site does not play an important role in normal physiological expression of the ANP gene. Recent studies have also demonstrated that constitutive expression of c-*fos* cannot activate ANP gene expression in cotransfection studies, and they suggest that other candidate transcription factors such as Egr-1 may be involved in the inducible expression of the ANP gene (Knowlton et al., 1991). Analysis of the sequences of the promoter region has revealed the presence of potential serum-responsive element within the ANP promoter (Knowlton et al., 1991). Interestingly, the sequences that contained the serum-responsive element were required for efficient transcription by stretching of the c-*fos* gene, and this inducible expression of c-*fos* appeared to be mediated by activation of PKC (Komuro et al., 1991). Thus, it remains to be established whether a serum response element, analogous to the c-*fos* gene, may be involved in transducing the mechanical signal into changes in ANP gene expression.

Transgenic models have been valuable in studying the role of specific genes in the growth and development and are now used to study the role of specific *cis* elements and *trans*-acting factors in the basal and inducible gene expression. Field (1988) reported that 472 bp of human ANP 5′-flanking sequences were sufficient to confer atrial specific expression upon the SV40 large T-antigen following the introduction of the chimeric gene (*hANP-Tag*) into transgenic mice. This study further delineated that these sequences promoted atrial specific expression, because expression of the oncogene by transgenic animals produced marked hyperplasia only of the right atrium, whereas the left atrium remained normal in size.

To determine whether these sequences would also be sufficient to confer inducible expression during ventricular hypertrophy, thoracic banding (7 days) was induced in a series of mice derived from the ANP-T-antigen transgenic line (Rockman et al., 1991). As assessed by Nothern blotting, thoracic banding produced a marked induction of endogenous ANP gene, but transgene expression was not significantly induced in the ventricle (Rockman et al., 1991). These results suggest that the tissue-specific and -inducible expression of the ANP gene can be segregated and that a distinct set of regulatory *cis* sequences (between −638 and −500 of the human and rat ANP 5′-flanking region) may mediate the up-regulation of the ANP gene during in vivo pressure overload hypertrophy. Similar results were seen in studies in which a series of rat ANP luciferase fusion genes was used to identify a 315-bp *cis* regulatory sequence (between −638 to −323 bp), which appeared to mediate the α_1-adrenergic inducible expression in neonatal ventricular myocytes (Knowlton et al., 1991). In contrast, when rat an ANP-CAT construct containing 3412 bp of ANP 5′-flanking sequence was transfected to neonatal ventricular myocytes, there was no induction of CAT activity by static stretch for 48 h (Sadoshima et al., 1992), suggesting that DNA sequences necessary for inducible ventricular expression may be located outside of this 3.4-kb sequence.

In conclusion, although several important advances have been made in understanding the potential signaling mechanisms that activate the ANP gene expression, the molecular mechanisms regulating ANP gene expression, particularly in response to pressure and volume overload, are not yet known. The mechanical as well as several autocrine and paracrine ANP gene-activating stimuli must generate signal(s) that reach the nucleus and activate the transcription of the ANP gene. Results of recent studies suggest that the activation of PKC may represent one of the most proximal common signaling events for both the ANP release and ANP gene expression. Although the mechanisms that mediate cardiac specific and inducible expression of the ANP gene can be segregated, specific sets of regulatory elements that mediate inducible ANP gene expression remain to be clarified. Finally, the magnitude of induction of ANP mRNA levels, coupled with the relatively low basal ANP expression in the adult ventricle, provides one of the best available molecular markers to monitor signaling pathways for transcriptional activation during ventricular cell hypertrophy.

IV. Regulation of Atrial Natriuretic Peptide Secretion

Endocrine systems are commonly activated by a stimulus or stimuli to release a hormone that acts on a distal target to elicit responses. These responses induce negative feedback, diminishing the stimulus and thereby additional hormone release. Thus, proof that ANP functions as an endocrine system required demonstration of the appropriate stimuli for ANP release from the heart into the circulation. The mechanisms controlling ANP release have been the subject of intense research and are now fairly well understood, although the complex interplay between mechanical and other factors have made their overall relative contributions hard to discern. Most attention has focused on the role of atrial pressure as a stimulator of ANP secretion, and it is now clear that an increase in atrial stretch is the major regulator of ANP secretion. Several physiological manipulations have been identified that acutely increase the circulating levels of ANP, particularly those perturbations, such as volume expansion and water immersion, that increase atrial wall tension. However, a number of extracellular signals in addition to mechanical stretching seem to be involved in regulating ANP secretion, including the rate of contraction, endothelial factors, peripheral and central nervous systems, hypoxia, and osmolality, as well as multiple neurohumoral factors (table 3).

A. Methods Used in the Study of Atrial Natriuretic Peptide Secretion

A variety of model systems have been used in studies of ANP secretion, including human and animal studies in vivo, isolated heart, isolated atria, and isolated cells or primary cardiac myocyte cultures. All models have provided valuable information from the secretory process, if appropriately validated. However, each model sys-

TABLE 3
Factors that have been shown to affect plasma ANP levels in vivo or stimulate ANP secretion in vitro

Stretching of myocytes
Volume expansion
Sodium intake
Water deprivation
Hemorrhage
Water immersion
Posture
Heart rate
Neurohumoral agonists
Epinephrine, norepinephrine
Acetylcholine
Vasopressin
Angiotensin
Endothelium-derived contracting and relaxing factors
Endothelin
EDRF
Arachidonic acid and its metabolites
Peripheral and central nervous system
Exercise
Osmolality
Hypoxia and hypercapnia
Myocardial ischemia and metabolic changes
Peptides
Opiates
CGRP
Other stimuli

tem also has several limitations, and some of these will be summarized briefly.

1. In vivo experiments. Most commonly, intact animals or human subjects have been used to study the factors that regulate ANP secretion. Human and intact animal studies are often difficult to interpret because the different stimuli themselves invoke a complex array of hemodynamic responses, neuronal reflexes, and changes in circulating humoral factors. These concomitantly occurring secondary alterations to a given stimulus may affect pressure and volume status and, thus, ANP release. Studies in which intact animals and human subjects are used are also complicated by the fact that arterial and venous IR-ANP concentrations result from cumulative changes in the secretion rate, elimination rate, and ANP distribution volume. Thus, measurement of the absolute secretory rate of ANP in vivo in response to different stimuli would require the analysis of the coronary sinus blood and the measurement of coronary (atrial) blood flow. Because these are difficult to obtain, most investigators have taken the plasma IR-ANP concentration in the arterial plasma as a measure of cardiac ANP release in response to different stimuli in vivo. In addition, the measurements of atrial and ventricular myocyte levels of ANP and ANP mRNA together with assay of plasma IR-ANP levels are more informative, because the interpretation of a single plasma measurement in response to chronic stimulation is complex. For example, if the plasma IR-ANP concentration increases in response to atrial distension induced by volume expansion and is followed by decreases in blood pressure and intravascular volume, plasma IR-ANP should decrease, despite the continued presence of the stimulus for the release of ANP; thus, "normal" plasma IR-ANP concentration does not necessarily exclude the possibility that ANP release from the heart is chronically stimulated.

The release of ANP to a given stimulus appears to be quantitatively dependent on the volume/pressure status (distension) before the stimulus, because atrial stretch is a major stimulus for ANP release. Thus, with low atrial pressures or low levels of resting tension (diastolic filling pressure), ANP secretion to some hormonal factors and tachycardia appears to be lower than the ANP secretory response at normal and high levels of resting wall tension. In fact, the release of ANP under these physiological circumstances (decreased intravascular volume and/or hypotension) would be a physiologically inappropriate response. The volume and pressure status should be thus reported and kept constant in experiments in intact animals and humans. Furthermore, different anesthetics have been reported to influence plasma ANP levels (Gutkowska et al., 1984a; Horky et al., 1985; for review, see Genest and Cantin, 1988), and conscious animals respond differently from anesthetized preparations. The measured changes in plasma IR-ANP levels induced either by physiological stimuli or experimental manipulations may also be confounded by variabilities among radioimmunoassays, plasma extraction procedures, and the source of the antibody used and its cross-reactions with COOH- and NH_2-terminal peptides and degradation products (for review, see Cernacek et al., 1988b).

2. Isolated perfused heart and isolated atria preparations. The in vitro models allow the study of ANP release in the absence of factors such as hemodynamic changes, circulating hormones, or central neuronal pathways. Most in vitro studies that have determined the effects of different stimuli on ANP release have been conducted in isolated atrial strips under some initial tension. In this model, the resting tension and the atrial contraction rate can be kept constant. In these studies, the amount of ANP in the bathing fluid has commonly been measured after a number of minutes of stimulation and has been attributed to leakage from the atrial tissue. The release that occurs from the atrial tissue must go through either the capillary wall or endocardium and down a concentration gradient into the bathing fluid. ANP secretion may also come from the cut edge of these tissues and move into the bathing fluid. These mechanisms of ANP extrusion may also occur in studies using atrial minces, fragments, slices, or pieces that are not under any basal tension. The preparation method appears to be critical when isolated atria are used (Sonnenberg et al., 1989).

In the normal Langendorff model, the atria do not fill, because they are open to the air, and thus the effect of atrial stretch is minimized, enabling studies of ANP release. The working heart may present a more physiological profile than other in vitro preparations with regard to normal function of the myocytes in that it is beating, the tissues are intact, and the chambers of the heart can be uniformly distended by pressures approximating those found in vivo. The preparation is not subject to neural influences or uncontrolled changes in stretch. However, it is subject to hemodynamic alterations resulting from chemically induced changes in myocardial contractility or heart rate, which should be kept constant and/or carefully measured. In the isolated perfused heart, ANP is released physiologically into the coronary venous circulation, and the preparations permit the collection of samples at short intervals so that transient changes promoted by stimuli can be easily detected.

3. Cell cultures. In several laboratories, primary cultures of atrial and ventricular myocytes have been utilized to study ANP secretion. During dissociation for cell culture, the ultrastructure of the heart tissue is lost, and isolated cells may not be able to synthesize, process, and release ANP in a similar manner to that occurring under normal physiological conditions. Furthermore, for technical reasons, most primary myocyte culture systems developed for biochemical studies utilize neonatal rat tissue, and it has been argued that the neonate may not be capable of secreting and processing ANP. Several reports have shown that cultured neonatal or adult atrial

myocytes, maintained in serum-free media that lack proteases, mainly secrete $proANP_{1-126}$, although when perfused, neonatal rat hearts, like adult hearts, secrete ANP_{99-126}. Cultured myocytes derived from ventricular tissue also primarily secrete $proANP_{1-126}$. Furthermore, the observation that the simple addition of protein alone into the incubation medium stimulates IR-ANP secretion from neonatal cultured cells (Sylvestre et al., 1986) shows the importance of validation of the method used for studying ANP secretion. The cell cultures permit the study of ANP release in the absence of most factors complicating in vivo and other in vitro models, including hemodynamic factors, circulating hormones, and neuronal pathways. However, either the rate of contraction is generally slow or isolated cells do not contract as they do in vivo. In addition, even if the cells are rhythmically depolarized and their length changes during each contraction in cell culture, stimuli may still have indirect effects upon the rate of contraction or tension and thus upon ANP secretion. In summary, the differences between model systems used are highly important when attempting to understand the conflicting results of different studies.

B. Effect of Stretching of Myocytes

The view that mechanical stretch of atrial tissue might induce ANP release was based on early studies of the relationship between atrial distension and diuresis. Inflation of a balloon within the left or right atrium results in enhanced urine flow and salt excretion (Henry et al., 1956; Baisset and Montastruc, 1977). However, expansion of extracellular fluid volume fails to elicit an increase in urine in the absence of atrial stretch (Goetz, 1975). The renal response provoked by atrial distension has usually been ascribed to reflex stimulation of neural stretch receptors, but a humoral mediator has also been considered (for reviews, see Gauer and Henry, 1963; Goetz, 1975; Linden, 1979; Hainsworth, 1991). Considerable increases in venous return and atrial volume in heart-lung preparations caused the release of a substance that increased urine flow and fractional sodium and fractional potassium excretion when injected into intact rats, thus supporting the presence of a blood-borne factor that mediates diuresis and natriuresis (Dietz, 1984).

1. In vitro models. Lang and his colleagues showed conclusively that ANP is released from the atrium in response to stretch (Lang et al., 1985; Ruskoaho et al., 1986a). The experiments were made in isolated perfused rat hearts by means of a modified Langendorff preparation, in which a change in intraatrial pressure is equal to transmural pressure and thus atrial distension. The right atrial pressure could be maintained constant at any desired level by adjusting the perfusion flow through the right atrium (Lang et al., 1985; Ruskoaho et al., 1986a). Increasing the perfusion rate produced an increase in right atrial pressure, which was accompanied by an increase in IR-ANP released into the perfusate. The pressure required (0.4 to 4.5 mm Hg) for ANP release was within the physiological range (Ruskoaho et al., 1986a). The isolated perfused heart is surrounded by atmospheric pressure, and the transmural pressure gradient is equal to the pressure within the atrium; thus, atrial stretch occurs with increased atrial pressure. The close linear, highly significant correlation observed between right atrial pressure and ANP release indicates that stretch determines the rate of ANP secretion.

The material released before and during stretch, analyzed by high-pressure liquid chromatography, was found to elute as a single peak at the position of synthetic ANP_{99-126} and to be identical with circulating material in the plasma of intact, volume-expanded rats. Furthermore, perfusate from hearts subjected to atrial distension caused relaxation of vascular smooth muscle preparations previously contracted with noradrenaline (Ruskoaho et al., 1986a). Thus, these experiments show that atrial stretch causes release of active ANP and that no enzymatic conversion in circulation is required.

Other studies in isolated perfused hearts have confirmed that right (increase in preload) or left (increase in afterload) stretch alone is a major factor regulating ANP release from the heart (Kabayama et al., 1987; Dietz, 1987, 1988; Onwochei et al., 1987; Ito et al., 1988; Onwochei and Rapp, 1988, 1989; Synhorst and Gutkowska, 1988; von Harsdorf et al., 1988; Katoh et al., 1990; Mäntymaa et al., 1990; Ruskoaho et al., 1990; Toki et al., 1990). Repeated volume expansion or increase in preload produced a reproducible increase in the atrial pressure and IR-ANP release (Kabayama et al., 1987; Onwochei et al., 1987). Of note, the rabbit heart released more ANP during left atrial than during right atrial distension, which might be expected because the left atrial IR-ANP concentration in the rabbit is higher than the right (Synhorst and Gutkowska, 1988).

Extensive studies in other in vitro models including isolated atria and isolated myocytes have confirmed the results obtained in isolated perfused rat heart and heart-lung preparations. Schiebinger and Linden (1986a) measured IR-ANP release while the resting tension of superfused rat left atria was adjusted. In nonpaced and paced atria, 3- to 5-fold increases in resting tension resulted in a 35% increase in IR-ANP release, whereas lowering the tension by 50% decreased IR-ANP release 24 to 30%. Thus, beating was not necessary for stretch-induced ANP release, and the effect was probably not mediated by release of endogenous neurotransmitters, because stimulated secretion did not change after pharmacological blockade by the combination of propranolol, phentolamine, and atropine (Schiebinger and Linden, 1986a). Consistently, when isolated rat atria were distended by inflation of miniature balloon catheters, the concentration of IR-ANP in the medium increased similarly in proportion to the degree of stretch both from

left and right atria; this increase in ANP release is temperature dependent (Bilder et al., 1986), as also was confirmed later (Agnoletti et al. 1990c; Page et al., 1990). Results of other studies of isolated rat atria have supported these observations showing that atrial stretch increases IR-ANP release (de Bold et al., 1986; Agnoletti et al., 1987, 1990c, 1992; Ishida et al., 1988; de Bold and de Bold, 1989, 1991; Schiebinger, 1989; Page et al., 1990, 1991a,b; Kuroski-de Bold and de Bold, 1991). Mechanical stretching of the atrium led to an active and immediate movement of a substantial part of the granule population to the periphery of the cell (Agnoletti et al., 1989). In contrast to other results, when inverted isolated rabbit or rat atria were distended, the increase in IR-ANP release into the medium did not occur during distension but only after the reduction of the distension (Cho et al., 1988a,b, 1990, 1991; Seul et al., 1992). Atrial distension in this experimental model was suggested to first induce ANP release into the extracellular space and then into the lumen with a reduction in atrial distension (Cho et al., 1990).

The results obtained using isolated myocytes are consistent with the observations in isolated perfused heart and isolated atria. Osmotic stretch of the plasma membrane of neonatal atrial and ventricular myocytes induced by hypotonic incubation media was a potent stimulus for IR-ANP secretion (Greenwald et al., 1989). Preliminary experiments in which flexible-bottomed collagen-coated culture dishes were used have also showed that cyclic or constant mechanical stretching of neonatal myocytes causes an increase in IR-ANP release into the incubation medium (Gardner et al., 1991; Tokola et al., 1991a). These data thus show that atrial distension alone is a major stimulus for ANP secretion and the mechanism whereby changes in intravascular volume or blood pressure are responsible for the alterations in hormone level.

2. *In vivo studies.* a. VOLUME EXPANSION. The above observations in vitro have been supplemented by numerous reports indicating that intravascular volume expansion in experimental animals or human subjects increases the concentration of IR-ANP in plasma. In vivo, changes in atrial pressure correlate positively with circulating IR-ANP levels under many experimental, physiological, and pathophysiological conditions. Lang and colleagues (1985) first reported that increases in atrial pressure resulted in enhanced release of ANP. The mean plasma concentration of IR-ANP in pentobarbital-anesthetized rats before volume expansion was 55 pg/ml. Plasma levels of IR-ANP showed marked and dose-dependent increases on volume expansion (Lang et al., 1985). Infusion of 2 ml of 0.9% saline within 1 min produced a 2- to 3-fold increase in plasma IR-ANP concentration. Administration of 8 ml, corresponding to about 30% volume expansion, gave a 6-fold increase. The peak right atrial pressure observed at the end of the infusion in these experiments was 1 to 1.5 and 5 mm Hg, respectively (Lang et al., 1985). Thus, ANP is a circulating hormone stimulated by volume loading.

Subsequent studies in intact animals, as well as in humans, using different stimuli have established a positive correlation between atrial pressure and circulating IR-ANP. In rats, acute volume expansion induced by isotonic saline or Ringer's solution infusion causes an increase in plasma IR-ANP concentration (John et al., 1986; Kato et al., 1986a; Kihara et al., 1986; Stasch et al., 1986; Chiu et al., 1987; Fried et al., 1987; Kaneko et al., 1987; Khraibi et al., 1987; Kohno et al., 1987a; Hansell et al., 1988). A significant increase in plasma IR-ANP concentration in anesthetized or conscious rats has also been shown in response to acute volume expansion with whole blood (Anderson et al., 1986a; Hirth et al., 1986; Pettersson et al., 1986; 1988; Barbee and Trippodo, 1987; Garcia et al., 1987b; Chien et al., 1988; Paul et al., 1988; Hebden et al., 1990), isooncotic albumin (Schwab et al., 1986b; Hebden et al., 1990), polygelene colloidal solution (Anderson et al., 1986a), or 5% glucose solution (Kato et al. 1986a). When measured, the increase in plasma IR-ANP concentration has been shown to correlate with central venous pressure (Pettersson et al., 1986; Schwab et al., 1986b), right atrial pressure (Barbee and Trippodo, 1987; Fried et al., 1987), or left atrial pressure. In two studies, the inflation of a balloon in the inferior vena cava (Fried et al., 1987) or a caval snare (Barbee and Trippodo, 1987) limited the volume expansion-induced increase in right atrial pressure, and this was accompanied by a significantly lower plasma IR-ANP level compared with rats whose right atrial pressure was allowed to increase.

Experiments in other species have confirmed that the plasma IR-ANP concentration increases in response to a volume load. Induction of volume expansion produced a 3-fold increase in plasma IR-ANP concentration in anesthetized rabbits (Wei ei al., 1986). Similarly, in anesthetized normal or nephrectomized rabbits, volume expansion produced by saline infusion increased central venous pressure and plasma IR-ANP levels (Volpe et al., 1988b, 1989). In conscious dogs, the plasma IR-ANP concentration increased transiently 2.4- to 2.5-fold above control values after 30 min of volume expansion with isotonic saline and then remained elevated for an additional 30 min (Salazar et al., 1986b; Verburg et al., 1986). The ingestion of a high sodium meal (125 mEq of sodium), which was calculated to result in postprandial expansion of extracellular fluid volume by 9%, increased plasma IR-ANP levels by 45% within 240 min (Verburg et al., 1986), further showing that the concentration of IR-ANP in plasma is reflective of changes in the intravascular volume status in the conscious dogs. In pentobarbital-anesthetized dogs, acute saline volume expansion (3.3, 6.6, and 10%) caused an increase in right atrial and pulmonary wedge pressure and peaked midway

through volume expansion before decreasing during stable volume expansion (Zimmerman et al., 1987b). Circulating levels of IR-ANP paralleled the increases and decreases in right and left atrial filling pressures (Zimmerman et al., 1987b). In response to volume loading in conscious dogs, the occurrence of the peak plasma IR-ANP level (unextracted) lagged 10 min behind the peak level of mean left atrial pressure, and a linear correlation between the increase in mean left atrial pressure and circulating IR-ANP level was noted (Nishida et al., 1988b). The experiments by Akabane et al. (1988) show the influence of basal right atrial pressure in stimulated release of ANP. When right atrial pressure in anesthetized dogs was increased by adjusting the height of a reservoir (connected to right atrium) from 2.7 to 9.0 mm Hg, plasma IR-ANP concentration did not increase significantly. However, if right atrial pressure was increased from 9.0 to 17.0 mm Hg, IR-ANP levels were significantly increased, and a positive correlation between right atrial pressure and IR-ANP release was found (Akabane et al., 1988). When the effects of five different volume expansion protocols in anesthetized dogs were compared, saline (0.9%), iso- and hyperoncotic dextran (4 and 25%), and homologous plasma caused the expected increase in plasma IR-ANP concentration, whereas iso- and hyperoncotic bovine serum albumin caused little or no increase in plasma IR-ANP levels (Cernacek and Levy, 1991). Plasma IR-ANP concentration closely correlated with central venous pressure; during bovine serum albumin expansion, the lack of atrial response was related to the absence of increment of central venous pressure, presumably because of histamine release with subsequent extravasation of fluid from capillaries (Cernacek and Levy, 1991). Thus, plasma IR-ANP may not be increased if the extra volume is not proportionately centralized and, therefore, does not cause an elevation of atrial pressure.

As in other mammalian species, circulating levels of ANP in humans are elevated when intravascular volume or right atrial pressure is increased (Sagnella et al., 1985; Shenker et al., 1985; Weil et al., 1985; Yamaji et al., 1985b; Anderson et al., 1986b; Kimura et al., 1986; Nishiuchi et al., 1986; Sato et al., 1986; Singer et al., 1987; Sugawara et al., 1988a; Tulassay et al., 1988; Yamasaki et al., 1988; for reviews, see Weidmann et al., 1989; Sagnella and MacGregor, 1990). These observations are also supported by the study of Schwab et al. (1986a) which showed that an increase in right atrial pressure in patients supported with the artificial Jarvik-7 heart results in an elevation of plasma IR-ANP concentration. On the other hand, decreased plasma IR-ANP levels have been reported in human subjects with edematous states associated with decreased circulating fluid volume (for review, see Cody, 1990) or after furosemide administration (Kimura et al., 1986; Yamasaki et al., 1988). The increase in plasma IR-ANP concentration in humans in response to acute volume expansion or an increase in dietary sodium intake may be relatively small (for reviews, see Blaine, 1990; Goetz, 1990). For example, administration of 2 liters of saline i.v. to supine human subjects during 2 h increased plasma IR-ANP levels by about 30% (Yandle et al., 1986a). Circulating levels of IR-ANP are also elevated during normal pregnancy (Cusson et al., 1985), although conflicting results have also been reported (for a recent review, see Fournier et al., 1991).

In most of the above-mentioned studies, measurements of elevation of mean right or left atrial pressure was used as an index of increased atrial stretch. However, mean atrial pressure does not accurately reflect atrial filling in response to volume expansion or other stimuli. During contraction (a wave) atrial pressure increases as a result of atrial contraction, whereas diameter decreases as blood is pumped into the ventricle. During atrial diastole (v wave), the atrium is passively filling; therefore, both pressure and diameter increase in parallel. Hintze et al. (1989) measured the effect of acute volume expansion (1000 ml of saline within 5 min) on mean arterial pressure, heart rate, left atrial systolic and diastolic dimensions, and pressures in chronically instrumented conscious dogs. a-wave left atrial diameter increased by 6% and v wave diameter by 13% during volume expansion, indicating an increase in atrial end-systolic and end-diastolic dimensions. Volume expansion increased mean left atrial pressure by 206% and caused a 663% increase in plasma IR-ANP concentration (Hintze et al., 1989). However, during volume expansion, both left atrial wall stress and minute wall stress increased to a significantly greater extent than did mean left atrial pressure and thus would be more sensitive indices of atrial stretch. The results were based on a number of assumptions to derive atrial wall stress; in conscious dogs, volume expansion increased heart rate and mean arterial pressure, whereas right atrial pressure and dimensions were not measured. Nevertheless, in the conscious dog, passive stretch of the left atria occurred mainly during the γ-wave of atrial cycle, and at high distending pressures, minute atrial wall stress gave the best estimates of the stimulus for ANP secretion.

Atrial appendectomy reduces the increase in plasma IR-ANP concentration induced by volume loading in experimental animals showing that ANP is mainly released from atrial appendages in response to acute volume loading. Villarreal et al. (1986) showed that atrial appendectomy suppressed the increase in plasma IR-ANP concentration produced by acute volume loading in the anesthetized rat. Similar results in rats have been reported by others after unilateral right (Schwab et al., 1986b; Garcia et al., 1987b) or bilateral (Hoffman et al., 1990; Hoffman and Keiser, 1990) atrial appendectomy. In the monkey, bilateral appendectomy greatly attenuated the increase in plasma IR-ANP concentration and the natriuresis after acute volume expansion (Benjamin

et al., 1988). The effects of atrial appendectomy on plasma IR-ANP levels in dogs have been more controversial (Benjamin et al., 1987; Cowley et al., 1988). Yet, in a recent study in conscious dogs by Stewart et al. (1992), a rapidly administered large volume load consistently produced a significant increase in mean left atrial pressure, mean arterial pressure, and plasma IR-ANP concentration. There were no significant differences in hemodynamic effects of volume loading after bilateral atrial appendectomy. Plasma IR-ANP concentration did not increase above baseline levels in atrial appendectomized dogs during volume loading, and natriuresis and diuresis were also reduced by 50% after appendectomy (Stewart et al., 1992). These studies show that atrial appendages greatly contribute to plasma IR-ANP concentration and natriuresis and diuresis during acute volume loading.

b. DISTENSION EXPERIMENTS. In vivo, mitral or tricuspid obstruction or pulmonary and aortic constriction have been used to provide stretch of the atrial wall. Mitral valve obstruction induced by inflating a left atrial balloon increased left atrial pressure by 11 cm H_2O and plasma IR-ANP concentration significantly (from 97 to 135 pg/ml) in chloralose-anesthetized dogs (Ledsome et al., 1985). Bilateral cervical vagotomy or vagotomy and administration of atenolol did not prevent the increase in plasma IR-ANP levels, showing that the release was probably due to atrial stretch (Ledsome et al., 1986). Observations of the time course of the changes in plasma IR-ANP concentration during partial mitral obstruction showed that IR-ANP was increased after 2 min of atrial distension and reached a steady value within 5 min (Ledsome et al., 1986). Similarly, distension of either the right (mitral stenosis) or the left atrium (tricuspid stenosis) in conscious dogs (Goetz et al., 1986) caused an increase in circulating IR-ANP. For each 1 mm Hg increase in atrial pressure, plasma IR-ANP concentration increased acutely by about 30 to 45 pg/ml. The cardiac nerves were not required to elicit an increased ANP release during atrial distension because the secretory response to distension of the left atrium in conscious dogs with chronic denervation did not significantly differ from the response observed in conscious dogs with intact cardiac nerves (Goetz et., 1986). Metzler et al. (1986) examined the effect of graded increments in right or left atrial volume by inflating a cuff around the pulmonary artery or ascending aorta in conscious dogs. They found that increases in either right or left atrial pressure within the normal physiological range is sufficient to increase plasma IR-ANP levels. Other studies have confirmed these observation in anesthetized dogs (Akabane et al., 1987; Diringer et al., 1992).

In a recent study, temporal changes in plasma IR-ANP concentrations in response to controlled increases in atrial pressure produced by an externally adjustable occluder around the pulmonary artery was examined in conscious dogs (Shin et al., 1991). When atria were rapidly stimulated by distension, the ANP secretion response was greatly attenuated within a few hours, and several days were required before ANP secretion returned to a level comparable to that achieved under acute conditions (Shin et al., 1991). Results of this study suggest that ANP secretion does not chronically adapt (within 7 days) to stimulation by increased atrial pressure.

c. CARDIAC TAMPONADE. The relative roles of atrial pressure versus atrial stretch as mediators of ANP release have been studied in intact animals by comparing the effect of acute cardiac tamponade with that of volume expansion or constriction of the great arteries. Cardiac tamponade represents a unique condition in which atrial pressure is acutely elevated; however, because of a balanced increase in both intraatrial and pericardial pressures, transmural atrial pressure (the pressure difference across the wall of a cardiac chamber) does not increase. Thus, in vivo distension can occur only as a result of increased transmural pressure. In anesthetized, open-chest dogs, unrestricted volume expansion increased right atrial and left ventricular end-diastolic pressure and caused a significant increase in plasma IR-ANP concentration (Mancini et al., 1987). Elevations of right atrial pressure caused by tamponade were comparable to those induced by volume infusion, but an increase in plasma IR-ANP concentration was not elicited during tamponade, and the ANP response to volume loading was abolished when performed during tamponade. The correlations of changes in plasma IR-ANP concentration and right atrial pressure or left ventricular end-diastolic pressure were significant only in the absence of tamponade consistent with a stretch-dependent mechanism of ANP release (Mancini et al., 1987). In a subsequent study, Edwards et al. (1988b) showed that right and left atrial transmural pressures did not change significantly during tamponade, whereas both pressures increased significantly with constriction of the pulmonary artery and aorta in anesthetized dogs. Plasma IR-ANP concentration did not change during cardiac tamponade but increased significantly in association with great artery constriction (Edwards et al., 1988b). The results of other workers confirm these findings in anesthetized dogs (Stone et al., 1989; Zioris et al., 1989) and pigs (Hynynen et al., 1990). In the latter study, plasma IR-ANP levels decreased in association with the decreased left and right atrial transmural pressures, and after release of tamponade, plasma IR-ANP levels increased. Finally, in conscious euvolemic dogs, increases in mean right atrial pressure in the absence of increased atrial size during tamponade are not associated with increased plasma IR-ANP levels (Klopfenstein et al, 1990).

In support of the above experimental data, Koller et al. (1987) observed an inappropriately low plasma level of IR-ANP in individuals with elevated atrial pressure

secondary to cardiac tamponade. This failure of ANP to increase in response to elevated atrial pressure associated with cardiac tamponade may in part contribute to the acute sodium retention known to occur. Following pericardiocentesis, atrial dimensions increased as did plasma IR-ANP concentration (Koller et al., 1987). An increase in plasma IR-ANP levels also occurred in patients undergoing pericardectomy for chronic constrictive pericarditis (Wolozin et al., 1988) and in anesthetized dogs during volume expansion by pericardectomy (Stone et al., 1989). In the latter study, the plasma IR-ANP concentration (unextracted) began to increase when a transmural pressure of 3 to 4 mm Hg was attained and the atrium was distended to 80% of its maximal diameter (Stone et al., 1989), suggesting that the right atrium under these experimental conditions can be subject to considerable pressure before a significant strain is developed and ANP is released. These studies in vivo during cardiac tamponade thus confirm that atrial stretch is the principal stimulus for acute ANP release.

d. OTHER CONDITIONS LEADING TO INCREASE IN CARDIAC FILLING PRESSURE. Head-out water immersion under thermoneutral conditions is associated with a translocation of blood into the thorax that leads to a diuresis and natriuresis (Epstein, 1978). In addition, an expansion of plasma volume occurs that is brought about by a shift of fluid from the cellular compartment into the plasma compartment. Both in humans (Anderson et al., 1986c; Ogihara et al., 1986; Epstein et al., 1987; Pendergast et al., 1987) and experimental animals, including rats (Katsube et al., 1985) and dogs (Miki et al., 1986; Krasney et al., 1991), plasma IR-ANP levels are elevated during water immersion (for review, see Epstein et al., 1989). Katsube et al. (1985) reported that plasma IR-ANP levels increased about 2-fold in anesthetized rats during immersion. Immersion of seated human subjects in water up to their necks also elevated plasma IR-ANP levels approximately 2-fold (Epstein et al., 1987). Thermoneutral head-out water immersion, which increases atrial transmural distending pressures by 8 to 10 mm Hg, is a physiological stimulus for ANP release also in conscious dogs (Miki et al., 1986). When dogs were immersed, plasma venous and arterial IR-ANP levels increased by 129 and 121% from the preimmersion hydrated values and remained elevated for 200 min.

Head-down tilt induces a marked fluid shift from the lower to the upper half of the body and increases central venous pressure. A change from standing to the supine position thus slightly increases plasma IR-ANP concentration (Hodsman et al., 1985, 1986; Larose et al., 1985). Head-down suspension (antiorthostatic hypokinetic) produces a significant increase in plasma IR-ANP concentration in rats after 2 h of suspension (Gauquelin et al., 1987a). Acute physical exercise also stimulates the secretion of ANP in direct proportion to the intensity of exercise in humans (Hodsman et al., 1986; Nishikami et al., 1986; Raine et al., 1986; Somers et al. 1986; Tanaka et al., 1986; for reviews, see Freund et al., 1988; Miller et al., 1990b), rats (Ruskoaho et al., 1989a; Allevard et al., 1991), and dogs (Peronnet et al., 1989). The increase in circulating IR-ANP correlates with an increase in pulmonary wedge pressure during exercise on a bicycle ergometer in supine subjects. This finding suggests that the presumed increase in secretion of the peptide during exercise is attributable to atrial distension. However, other factors, including the increase in heart rate during exercise, may also increase the secretion of ANP (see section IV.C).

e. VOLUME CONTRACTION. The above consideration shows that increases in atrial filling pressure stimulate the secretion of ANP. Conversely, a decrease in atrial filling pressure should lead to a reduction in ANP secretion. Results from several studies indicate that both acute and chronic volume contraction induce decreases in plasma IR-ANP levels. Takayanagi et al. (1985) were the first to demonstrate that chronic water restriction causes a decrease in circulating IR-ANP levels. A progressive reduction of body fluids due to 3 days of water restriction decreased circulating IR-ANP to below detectable levels in normal conscious rats. In rats dehydrated for 72 h and then allowed to drink, 90% of the rats began to drink immediately when water was offered; plasma IR-ANP levels increased markedly within 3 min and gradually for up to 1 h after water was offered (Januszewicz et al., 1986b). Others have found that 1 (Chiu et al., 1987) or 5 days (Zisfein et al., 1986; Kohno et al., 1987a) of water restriction also caused a substantial reduction in plasma IR-ANP concentration. Administration of the loop diuretic ethacrynic acid to conscious dogs reduced plasma IR-ANP levels by 34% during the first 30 min after administration and remained 45% lower than the baseline level after 3 h (Verburg et al., 1986). The plasma IR-ANP concentration remained suppressed by 66% when measured 24 h after a 15% volume depletion (Verburg et al., 1986). The decline in the plasma IR-ANP concentration paralleled the rapid depletion of extracellular fluid volume and contraction of intravascular volume as measured by an increasing hematocrit. Similarly, in conscious rats, plasma IR-ANP levels were decreased significantly 1 (Chiu et al., 1987) or 5 (Kohno et al., 1987a) h after intraperitoneal administration of furosemide, which also resulted in significant volume depletion (Kohno et al., 1987a). In dogs with acute hypovolemic renal insufficiency induced by pancreatitis, plasma IR-ANP levels declined by half in association with contraction of the plasma volume and the decrease in central venous pressure (Levy and Cernacek, 1989). Thus, these results demonstrate a decreased circulating IR-ANP concentration in response to acute and chronic reductions in intravascular volume.

f. HEMORRHAGE. Because an increase in atrial stretch elevates the circulating level of ANP, one would expect

basal concentrations of ANP to be suppressed in response to decreased intravascular volume induced by hypotensive hemorrhage. In conscious animals and humans, however, hemodynamic and neurohumoral responses to acute hypovolemia are complicated and involve two distinct phases (Schadt and Ludbrook, 1991). During the first phase of acute blood loss, cardiac output and right atrial pressure decrease while blood pressure is well maintained by an increase in vascular resistance and heart rate. When central blood volume has been critically reduced, arterial pressure decreases because of a decrease in vascular resistance associated with withdrawal of sympathetic vasoconstrictor drive. The second phase is also characterized by absolute or relative bradycardia and an increase in release of adrenal catecholamines and vasopressin (Schadt and Ludbrook, 1991). These multiple factors, in addition to atrial stretch, influence the release of ANP, and thus, different secretory responses to contraction of blood volume may be expected depending on the phase studied.

Decreases in plasma IR-ANP levels in response to hemorrhage have been observed in conscious (Chiu et al., 1987; Garcia et al., 1987b; Morris et al., 1987a) and anesthetized rats (Phillips et al., 1989), anesthetized dogs (Edwards et al., 1988c; Geer et al., 1988), anesthetized rabbits (Courneya et al., 1989), unanesthetized swine (Carlson et al., 1989), and ovine fetuses (Cheung and Brace, 1991). This suppression of ANP release would thus protect the hypovolemic organism from further volume depletion. However, no change after hemorrhage has been reported in humans (Hodsman et al., 1986), dogs (Levy and Cernacek, 1989), and sheeps (Yates et al., 1992), and even increases after a large hemorrhage of 40% of the blood volume have been observed in conscious swine (Shackford et al., 1988) and dogs (Sakai, 1987). Increases in plasma IR-ANP levels have been reported also in endotoxic shock in dogs (Gullichsen et al., 1989) and in sheeps (Lubbesmeyer et al., 1988). Thus, in some circumstances, there may be no relationship between changes in blood volume (atrial dimensions) and changes in plasma IR-ANP concentration.

The acute decrease in plasma IR-ANP concentration after hemorrhage was related to decreased pulmonary wedge pressure (Edwards et al., 1988c), mean left atrial pressure (Geer et al., 1988; Wang et al., 1990), or right atrial pressure (Geer et al., 1988; Wang et al., 1990). However, the relationship between atrial pressure and plasma IR-ANP level is not evident during relatively slow, prolonged hemorrhage (Geer et al., 1988). Furthermore, in some pigs, IR-ANP concentration increased during the first hour after hemorrhage, and changes in IR-ANP concentration were unrelated to decreases in central venous pressure or absolute right atrial volume determined with a conductance catheter (Carlson et al., 1989). Thus, tachycardia, the release of other hormones in addition to ANP, or related factors may oppose the response of ANP to reduced atrial volume after hemorrhage. In support of this, plasma IR-ANP levels increased early after hemorrhage in some pigs, and these increases occurred most frequently at atrial rates >200 beats/min (Carlson et al., 1989). Furthermore, the correlation of changes between plasma IR-ANP concentration and atrial rate suggests that the tachycardia after hemorrhage opposes the effects of decreased blood volume (Carlson et al., 1989). The changes in plasma IR-ANP concentration also correlated significantly with norepinephrine, renin, and vasopressin levels, and the response of vasopressin proved the best predictor of the ANP response in multiple regression analysis (Carlson et al., 1989). Thus, it is possible that the increase in other circulating hormones may have acted to offset any inhibitory effect of a further decline on ANP secretion. On the other hand, a comparison of the ANP response to hemorrhage revealed no significant difference between the sham-operated and cardiac denervated dog, thus providing no evidence for a specific effect of cardiac nerves on ANP secretion during hemorrhage (Geer et al., 1988). Cutting the vagus nerve did not prevent the plasma IR-ANP response to hemorrhage in anesthetized rats (Phillips et al., 1989). The lack of response to blood volume reduction in anesthetized rabbits was explained by the slow rate of volume depletion (1%/min) (King and Ledsome, 1991; Ledsome and King, 1991). In conclusion, ANP response to contraction of blood volume is variable. Plasma IR-ANP levels decrease as atrial volumes acutely decrease, but there is no correlation between plasma IR-ANP levels and atrial pressure during prolonged hemorrhage. This may be explained by other factors affecting the release of ANP or decreased clearance (see section V).

g. EXPERIMENTAL MODELS LEADING TO DECREASE IN CARDIAC FILLING PRESSURE. The conscious dog with chronic thoracic inferior vena cava constriction has been used as an experimental model to examine whether acute and chronic reductions in atrial pressure are associated with lowered plasma IR-ANP levels. Constriction of the thoracic inferior vena cava resulted in decreased right atrial pressure or central venous pressure and plasma IR-ANP concentration (Freeman et al., 1987; Paganelli et al., 1988). During the chronic phase of thoracic inferior vena cava constriction (10 to 12 days), when atrial pressures have increased toward control pressures, plasma IR-ANP concentration remained suppressed. The release of the chronic thoracic inferior vena caval constriction in conscious dogs resulted in acute elevations in right and left atrial pressures associated with modest increases in plasma IR-ANP concentration (Paganelli et al., 1988). Thus, plasma IR-ANP concentration is physiologically regulated during states of low and high atrial filling pressure associated with caval constriction and release.

Mechanical ventilation with positive end-expiratory pressure, a procedure that reduces atrial transmural pres-

sure, as well as altering other cardiovascular variables (Goetz, 1975), has been reported to reduce plasma IR-ANP levels in patients with acute respiratory failure and in healthy human volunteers (Andrivet et al., 1988; for reviews, see Weidmann et al., 1989; Sagnella and MacGregor, 1990). The decrease in atrial transmural pressure presumably reduces ANP secretion in these experiments.

C. Rate of Contraction

1. Effect of heart rate on atrial natriuretic peptide release in vivo and in vitro. An increased frequency of atrial contractions has been shown to be an effective stimulus for the release of ANP both in vivo and in vitro. In humans, increased circulating IR-ANP has been observed during spontaneous (Espiner et al., 1985; Gutkowska et al., 1985; Schiffrin et al., 1985; Tikkanen et al., 1985b; Yamaji et al., 1985a) and paced tachycardia (Crozier et al., 1986, 1987a; Erne et al., 1987; Roy et al., 1987; Ellenbogen et al., 1988, 1989; Peuhkurinen et al., 1992). Circulating levels of IR-ANP have also been shown to increase during atrial tachycardia in rabbits (Rankin et al., 1986; Morris et al., 1987b) and dogs (Armstrong et al., 1986; Walsh et al., 1987; Christensen et al., 1988; Hirata et al., 1989b; Miller et al., 1990a; Nishimura et al., 1990, 1991). In humans, peak IR-ANP levels are approached after pacing for 5 min (Crozier et al., 1986; Ellenbogen et al., 1988, 1989). When the changes in ANP release during 4 h of sustained tachycardia were measured in chloralose-anesthetized dogs, plasma IR-ANP concentrations increased within 5 min, peaked at 30 min, and thereafter declined, reaching control values after 240 min of rapid ventricular pacing (Walsh et al., 1988).

Results of in vitro studies suggest chronotropic stimulation to be a primary effector for ANP release (Bilder et al., 1986, 1989b; Schiebinger and Linden, 1986b; Motomura et al., 1988; Wong et al., 1988a; Sanchez-Ferrer et al., 1990). Bilder et al. (1986) found that repetitive atrial distension caused a rate-dependent increase in IR-ANP release, which at the highest rate tested (89 inflations/min) was 6-fold higher than that seen during continuous maximal expansion. Increasing the frequency of pacing from 120 to 240 and 480 contractions per min resulted in about 46 and 190% increases, respectively, in the IR-ANP level over baseline in isolated rat atria (Schiebinger and Linden, 1986b). Right atria including the sinus node were also noted to spontaneously release greater quantities of IR-ANP than quiescent right atria (Wong et al., 1988a), and atrial tachycardia enhanced release of IR-ANP from the isolated blood-perfused dog right atrium (Motomura et al., 1988). Perfusion with 50 mM KCl caused cardiac arrest and a decrease in IR-ANP release to undetectable levels in the isolated rat heart (Naruse et al., 1987). Bilder et al. (1989b) further showed that chronotropic stimulation at controlled resting tensions >1 g increased IR-ANP release in a manner dependent on pacing frequency and that resting tension influenced the magnitude of the secretory response. Furthermore, IR-ANP was positively correlated with systolic tension at a given diastolic tension and pacing frequency (Bilder et al., 1989b). Cardiac pacing also increased IR-ANP release from isolated perfused rat heart (Doubell, 1989a,b). In contrast, some authors have reported that an increased rate had no effect on basal IR-ANP release from isolated rat atria (Agnoletti et al., 1990c, 1992), isolated rat heart (Katoh et al., 1990), or rabbit atria (Cho et al., 1991). However, electrical stimulation with atrial distension resulted in an increase in IR-ANP secretion in proportion to pacing frequency (Cho et al., 1991).

2. Mechanisms of tachycardia-induced atrial natriuretic peptide release. In vivo, the tachycardia-induced ANP secretion has been generally explained by an elevation of mean atrial pressure, because changes in atrial pressure or tension accompany paroxysmal tachycardia, intracardiac pacing, or electrical stimulation of isolated atria. Right atrial pacing increased the plasma concentration of IR-ANP with a simultaneous increase in mean right atrial pressure in anesthetized, vagotomized rabbits (Rankin et al., 1986, 1987a). In anesthetized dogs during atrial pacing, there was an increase in plasma IR-ANP concentration with the increase in mean right atrial pressure (Walsh et al., 1988c; Christensen et al., 1989a), pulmonary wedge pressure (Walsh et al., 1987, 1988b,c), or mean left atrial pressure (Hirata et al., 1989b; Ridder-vold et al., 1991). Similarly, rapid ventricular pacing in anesthetized dogs resulted in parallel increases in atrial pressure and plasma IR-ANP concentration (Walsh et al., 1988a; Miller et al., 1990a). In normal conscious dogs, atrial pacing increases plasma IR-ANP concentrations with marked elevation of mean left and right atrial pressures (Nishimura et al., 1990). Finally, increased heart rate in humans only increased circulating IR-ANP concentration when mean atrial pressure was increased (Roy et al., 1987).

Recent studies showed that the rate of contraction also stimulates the secretion of ANP in the absence of increased mean atrial pressure, and this has been used as an index of increased atrial stretch during tachycardia. In certain patients who underwent heart surgery and had normal cardiac function, plasma IR-ANP levels increased during atrial pacing at a mean rate of 130 beats/min without elevation of mean atrial pressure (Nishimura et al., 1986). In bilateral cervical vagotomized anesthetized rabbits, atrial pressures increased within the first 3 min of tachycardia, whereas the plasma IR-ANP concentration only reached statistical significance after 10 min (Rankin et al., 1987a). In pigs, plasma IR-ANP concentration increased during tachycardia, although low atrial pressure was maintained by controlled hemorrhage (Ziegler et al., 1987). An elevation of IR-ANP

concentration in anesthetized dogs persisted following tachycardia despite the absence of a persistent stimulus for elevated atrial pressure (Miller et al., 1990a). In conscious dogs with complete atrioventricular block, rapid atrial pacing (from 164 to 317 beats/min) significantly increased plasma IR-ANP concentration in spite of the lack of any appreciable changes in either mean left or right atrial pressure (Nishimura et al., 1990). These data suggest that tachycardia-associated ANP release might not be mediated by an increase in atrial pressure.

Direct measurements of atrial dimension changes by sonomicrometry have confirmed that the release of ANP during pacing tachycardia is not due to simple mechanical stretch of the atria. Tachycardia is thought to have little effect on atrial dimensions because atrial filling occurs during ventricular systole, the duration of which is only marginally affected by pacing at high rates. In anesthetized dogs, plasma IR-ANP concentration increased during pacing tachycardia without an increase in right atrial systolic or diastolic dimensions (Christensen et al., 1988). Similarly, in anesthetized rabbits, right atrial systolic and diastolic dimensions did not change during tachycardia, but plasma IR-ANP concentration increased significantly (King and Ledsome, 1990, 1991). In an open-chest dog model in which atrial stretch and heart rate were controlled and could be varied independently, the increase in heart rate from 150 to 250 beats/min with constant atrial dimensions caused a marked increase in plasma IR-ANP concentration, and the relationship between plasma ANP and left atrial dimensions was shifted upward by rapid pacing, i.e., plasma IR-ANP concentration was higher for any given atrial dimension (Riddervold et al., 1991). These results suggest that ANP release does not require a change in atrial stretch (atrial dimensions).

Thus, the increase in atrial wall tension/stress may be a more important determinant of ANP release than passive atrial distension. In support of this, Christensen et al. (1988) reported that in barbiturate-anesthetized, closed-chest dogs the product of systolic right atrial pressure and the frequency of contraction was more closely related to ANP release than to mean right atrial pressure or heart rate alone. The release of ANP in response to tachycardia also appeared to be most closely related to the minute index of systolic right atrial wall stress (Christensen et al., 1988; King and Ledsome, 1990, 1991). These results suggest that atrial systolic wall tension/stress represents an important stimulus to tachycardia-induced release of ANP.

A certain level of diastolic tension, however, is an important prerequisite for rate-induced release of ANP in vitro and in vivo. Bilder et al. (1989b) showed that resting tension influenced the magnitude of the secretory response and that IR-ANP release remained unchanged by electrical pacing when isolated rat atria were maintained at 1 g or less resting tension. In anesthetized dogs, an increase in pacing frequency had a much greater effect on plasma IR-ANP concentration at high (produced by blood volume expansion) than a low mean right atrial pressure (Christensen et al., 1988). Increasing the frequency of atrial contraction in anesthetized dogs with balloon occlusion of the inferior vena cava obstructing venous return reduced atrial pressure and wall tension but did not increase atrial pressure or plasma IR-ANP concentration. Alternatively, increased ANP secretion without inferior vena caval occlusion was related to the increased atrial pressure (Walsh et al., 1988b). Nishimura et al. (1990) noted that low atrial pressure was associated with a lower peak plasma IR-ANP level during atrial plus ventricular pacing in dogs with complete atrioventricular block. In anesthetized rabbits, tachycardia increased systolic right atrial pressure, and the increase at high blood volume was significantly greater than that at low blood volume (King and Ledsome, 1991). Furthermore, both systolic and diastolic atrial stress were significantly increased during pacing in the presence of atenolol, which alone increased diastolic right atrial stress (King and Ledsome, 1990). Thus, the effect of atrial pacing on ANP secretion may depend on the level of atrial resting (diastolic) dimensions, and systolic and diastolic function appear to act synergistically to increase ANP release during tachycardia.

It is unclear whether the release of ANP caused by the increased heart rate is modified by other factors accompanying pressure changes that modify atrial wall function, including several hormonal, vasoactive substances, or biochemical events that accompany tachycardia. The role of the autonomic nervous system in pacing-induced ANP release is still controversial. Rankin et al. (1986) reported that the release of ANP with tachycardia by atrial pacing was not influenced by administration of various neurotransmitter blockers (atenolol, atropine, and hexamethonium) in anesthetized rabbits. Although the stimulated secretion did not change after pharmacological blockade by the combination of propranolol, phentolamine, and atropine (Schiebinger and Linden, 1986b), the effect may be in part mediated by release of endogenous neurotransmitters, because the frequency-induced ANP release was reduced by reserpine pretreatment (Bilder et al., 1989b). Furthermore, the similar increase in plasma ANP in both control and cardiac denervated dogs (Williams et al., 1989) or vagotomized dogs (Hirata et al., 1989b) suggests that the secretion of ANP in response to pacing is independent of both sympathetic and vagally mediated central control. On the other hand, the secretory response appeared to be mediated by the autonomic nervous system in conscious dogs, because pacing-induced ANP release was suppressed after infusion of autonomic blocking agents (propranolol + phentolamine + atropine) (Nishimura et al., 1990). However, adrenergic antagonists cause changes in

wall stress/tension; therefore, ANP secretion may have been modified by lowered atrial pressure. Thus, the possibility that the autonomic nervous system contributes to tachycardia-induced ANP release remains to be established.

In conclusion, tachycardia stimulates ANP secretion from the heart, and the release is often associated with hemodynamic changes including increased mean atrial pressure. Thus, it appears that atrial stretch is the physiological determinant of ANP release. Recent experimental evidence, however, suggests that the increase in systolic atrial pressure and wall stress may also be an important determinant of ANP release as passive atrial distension. Moreover, systolic and diastolic function appear to act synergistically to increase ANP release during tachycardia, and the chronotropic stimulation both in vitro and in vivo is a more potent stimulator of ANP release at elevated diastolic tension. Although the physiological and pathophysiological importance of increased ANP release during tachycardia in vivo remains to be established, the dual stimulation of ANP release by atrial distension and the rate of contraction may be responsible for the high circulating ANP levels as well as natriuresis associated with paroxysmal tachycardia.

D. Effect of Neurohumoral Agonists

1. Epinephrine, norepinephrine, and other drugs acting on α-adrenoceptors. a. IN VITRO STUDIES. Although increased atrial wall stretch appears to be the major signal for the release of ANP, a variety of humoral factors have been implicated in controlling ANP secretion from the heart (for reviews, see Rankin, 1987; Ruskoaho et al., 1987, 1991; Goetz, 1988). Originally, Sonnenberg and Veress (1984) reported that incubation of isolated rat atria in vitro with 3 μM epinephrine increased the bioactivity of the incubation medium, tested by changes of renal function in anesthetized rats. Phentolamine, an α-adrenoceptor-blocking drug, largely abolished the natriuretic and diuretic effect of media incubated with epinephrine. In the isolated perfused rat heart, epinephrine at 10^{-6} to 10^{-7} M dose dependently increased heart rate and contractile force with a marked increase of IR-ANP in the perfusate (Ruskoaho et al., 1985; Toth et al., 1986). The ANP secretory response to epinephrine infusion was almost completely blocked by phentolamine and attenuated by metoprolol, a β-adrenoceptor antagonist (Toth et al., 1986). Epinephrine also caused a dose-dependent increase in IR-ANP release from dispersed atrial cells which could be antagonized by propranonol but not by phenoxybenzamine (Gibbs, 1987a,b). Addition of epinephrine to the perfusate induced a biphasic release of IR-ANP from the isolated rat right atria (Wong et al., 1988b); in this study, the initial peak could be inhibited by propranolol, and the sustained second peak could be inhibited by phentolamine. In contrast, epinephrine did not influence IR-ANP release from atrial tissue fragments or slices (Lachance et al., 1986; Naruse et al., 1986; Inoue et al., 1988a). Taken together, these data show that epinephrine stimulates ANP release from atria in vitro and that both α- and β-adrenoceptors appear to be involved in the effect of epinephrine on ANP release.

Most in vitro studies have shown that norepinephrine and other more selective α_1-adrenergic agonists directly stimulate ANP secretion (Currie and Newman, 1986; Schiebinger et al., 1987; Ishida et al., 1988; Onwochei and Rapp, 1988; Ruskoaho and Leppäluoto, 1988b; Wong et al., 1988b; Toki et al., 1990; Schiebinger et al., 1992; Schiebinger and Greening, 1992). Norepinephrine, an adrenergic agonist with both α and β properties, has been shown to stimulate IR-ANP secretion from isolated, perfused rat hearts (Currie and Newman, 1986). This effect appeared to be mediated via α-receptors, because it was blocked by phentolamine. Phenylephrine, a more selective α_1-adrenergic agonist also induced a dose-dependent increase in IR-ANP release into the perfusate, and phentolamine inhibited its secretory response (Currie and Newman, 1986). Superfusion of isolated left atria with norepinephrine produced a biphasic increase in IR-ANP secretion with a peak response 2.5-fold above baseline; both propranolol and phentolamine decreased the ANP secretory response to norepinephrine (Schiebinger et al., 1987). Phenylephrine in the presence of propranolol caused a monophasic increase in IR-ANP secretion from rat atria that was inhibited by phentolamine (Schiebinger et al., 1987). Phenylephrine stimulated IR-ANP release in heart-lung preparations independently of changes in atrial pressure, whereas clonidine, an α_2-agonist, failed to stimulate ANP release in isolated rat heart-lung preparations (Onwochei and Rapp, 1988). Methoxamine, another selective α_1-agonist, stimulated IR-ANP release from isolated rat atria (Wong et al., 1988b), perfused rat heart (Ruskoaho and Leppäluoto, 1988b), and isolated adult atrial myocytes (Hayashi et al., 1988). These results have been extended using atrial cardiocytes cultured in serum-free media. In this system, norepinephrine and phenylephrine stimulated IR-ANP secretion into the incubation medium (Matsubara et al., 1988a). The phenylephrine-stimulated IR-ANP secretion from atrial myocytes could be inhibited by prazosin (Shields and Glembotski, 1989). Additionally, norepinephrine could also stimulate ANP release; the norepinephrine-stimulated ANP release could be blocked by prazosin but not by propranolol (Shields and Glembotski, 1989). In some studies, norepinephrine had no effect on IR-ANP release in vitro (Lachance et al., 1986; Naruse et al., 1986; Gibbs, 1987b; Inoue et al., 1988a). Furthermore, norepinephrine did not significantly influence the ANP secretory response to stretch in the rat atria preparation (Schiebinger and Greening, 1992).

b. IN VIVO STUDIES. The administration of norepinephrine, phenylephrine, and α-adrenergic agonists produces an immediate increase in plasma IR-ANP levels

in vivo in rats (Katsube et at., 1985; Manning et al., 1985; Haass et al., 1987b; Baranowska et al., 1987a; Rankin et al., 1987b) and humans (Uehlinger et al., 1986, 1987; Sanfield et al., 1987; Tunny et al., 1987, 1988). Yet, in one study phenylephrine (and epinephrine) infused subcutaneously by osmotic minipump did not affect plasma IR-ANP levels (Garcia et al., 1986a). However, this phenylephrine dose did not affect blood pressure or any other variables studied. Whether α-adrenergic agonists only modify ANP release by changing atrial stretch (due to increase in blood pressure) or also directly stimulate hormone release in vivo is not clear. Katsube et al. (1985) gave phenylephrine to anesthetized rats and found that IR-ANP release closely parallels the elevations in atrial pressure (Katsube et al., 1985). Stewart et al. (1990) measured changes in plasma IR-ANP concentration and atrial wall function in conscious dogs after the administration of phenylephrine. They observed significant linear correlations between left ventricular afterload and left atrial pressure and concluded that ANP release in response to a bolus injection of phenylephrine is only dependent on the magnitude of atrial stretch under these experimental conditions. Similarly, changes in plasma IR-ANP in response to norepinephrine infusion (0.5 μg/kg/min for 30 min) correlated closely with the changes in left atrial pressure and to a lesser extent with the changes in right atrial pressure in conscious sham-operated and cardiac denervated dogs (Zhu et al., 1990). In support of these findings, the phenylephrine stimulatory effect on ANP release was completely abolished when its pressor effects were prevented by the additional infusion of nitroprusside in anesthetized rabbits (Volpe et al., 1991b). Thus, these studies suggest that changes in systemic arterial pressure caused by phenylephrine and other agonists stimulate atrial wall function and ANP release.

Yet, there is some evidence that the adrenergic stimulation may potentiate ANP secretion induced by an increase in atrial wall tension. Ruskoaho et al. (1989c) studied the effect of 30-min infusions of epinephrine on basal and volume expansion-induced plasma IR-ANP levels in pithed rats. When epinephrine was infused alone, no significant effect was seen on plasma IR-ANP concentration, whereas mean arterial and right atrial pressures increased significantly. In subsequent experiments, volume expansion in the presence of epinephrine induced significant increases in plasma IR-ANP concentration. The stimulatory response was less than in the presence of vasopressin, in spite of the fact that equipressor doses of both were used (Ruskoaho et al., 1989c). These data show that the release of ANP from the heart in response to atrial distension is hormonally modulated by epinephrine. In support of this, a 60-min i.v. infusion of 15 μg/kg/min phenylephrine in conscious rats produced at 30 min a 5-fold increase in plasma ANP in the absence of any increases in central venous pressure or left ventricular end-diastolic pressure (Lachance and Garcia, 1991d).

c. α_2-ADRENERGIC AGONISTS. Infusion of clonidine, an α_2-adrenergic agonist, either i.v. or i.c.v., into conscious rats produced a diuresis and marked increase in plasma IR-ANP levels (Baranowska et al., 1987a,b,c; Pan and Gutkowska, 1988; Chen et al., 1989). Yohimbine, an α_2-adrenergic antagonist, decreased plasma IR-ANP levels and partially inhibited the increase in IR-ANP induced by clonidine (Baranowska et al., 1987b; Pan and Gutkowska, 1988). The stimulatory effect of clonidine was also partially blocked by naloxone (Baranowska et al., 1987a; Pan and Gutkowska, 1988), whereas verapamil did not alter clonidine-stimulated increases in plasma IR-ANP concentration (Baranowska et al., 1987b). It is likely that this in vivo effect on circulating IR-ANP levels is the result of hemodynamic changes influencing atrial stretch because in vitro data have failed to provide evidence for a direct effect of an α_2-agonist on ANP release. In support of this, the infusion of medetomidine, a potent and selective α_2-agonist, significantly increased mean arterial blood pressure and right atrial pressure accompanied by an increase in plasma IR-ANP concentration in conscious normotensive rats (Ruskoaho and Leppäluoto, 1989), as well as in normal healthy volunteers (Kallio et al., 1989). The absence of an increase in plasma IR-ANP concentration in SHR in response to medetomidine was associated with a decrease in blood pressure (Ruskoaho and Leppäluoto, 1989), also suggesting that hemodynamic effects seem to explain the increase of ANP release in response to α_2-adrenoceptor stimulation.

2. Isoproterenol and other drugs acting on β-adrenoceptors. a. IN VITRO STUDIES. Studies of the effect of isoproterenol on ANP release in vitro have produced conflicting results. Incubation of isolated nonbeating rat atria in vitro with isoproterenol showed no release of IR-ANP (Sonnenberg et al., 1984; Naruse et al., 1986; Inoue et al., 1988a). Furthermore, β-adrenergic agonists did not stimulate IR-ANP release in incubated atrial slices (Lachance et al., 1986), and isoproterenol also failed to stimulate IR-ANP release in the isolated perfused rat heart (Currie and Newman, 1986) or heart-lung preparations (Onwochei and Rapp, 1988). Isoproterenol failed to stimulate IR-ANP secretion into the incubation medium from atrial cardiocytes cultured in serum-free media (Matsubara et al., 1988a). Iida and Page (1988) and Shields and Glembotski (1989) reported that isoproterenol inhibited IR-ANP release in noncontracting rat atrial myocytes. Furthermore, isoproterenol partially inhibited phenylephrine-stimulated IR-ANP secretion (Shields and Glembotski, 1989), suggesting that in atrial cells the stimulation of β-receptors inhibits ANP secretion.

In contrast, isoproterenol evoked IR-ANP release from perfused contracting rat atria (Schiebinger et al., 1987;

Wong et al., 1988b; Schiebinger, 1988, 1989; Schiebinger and Santora, 1989; Agnoletti et al., 1990c, 1992), from the isolated perfused spontaneously beating rat heart (Ruskoaho and Leppäluoto, 1988b) and from dispersed atrial myocytes (Gibbs, 1987b). In support of these results, the IR-ANP secretory response to epinephrine infusion was almost completely abolished by phentolamine and attenuated by metoprolol, a β-adrenoceptor antagonist (Toth et al., 1986). Continuous superperfusion of rat left atria with 0.1 μM isoproterenol resulted in a biphasic response similar to the secretory response to norepinephrine, and the stimulatory effect was inhibited by simultaneous superfusion with propranolol (Schiebinger et al., 1987). Importantly, superfusion of nonbeating, electrically quiescent left atria with isoproterenol failed to stimulate IR-ANP release, and in fact, IR-ANP secretion decreased (Schiebinger, 1989). When the atria were depolarized by KCl, isoproterenol did not cause the release of IR-ANP (Agnoletti et al., 1992). The difference in the response to β-adrenergic stimulation in beating and nonbeating isolated atrial preparations may be due to differences in the tension developed, because there is evidence that an increase in developed tension stimulates release of IR-ANP from isolated rat atria (Bilder et al., 1989b) and probably also explains the inability of β-adrenergic agonists to consistently induce ANP release observed in several in vitro experiments. Furthermore, the detection of a transient stimulatory effect is difficult using a metabolic chamber or incubation model.

b. IN VIVO STUDIES. Garcia et al. (1986a) were the first to report that isoproterenol (3 μg/kg/min), infused for 5 days subcutaneously by osmotic minipump in rats, produced a 3-fold increase in plasma IR-ANP levels with an increase in natriuresis. Systolic blood pressure did not change during the infusion, and heart rate and atrial pressure were not measured. Isoproterenol has been demonstrated to dose dependently increase plasma levels of ANP in the anesthetized, vagotomized rabbit (Rankin et al., 1987b). The time course of the response showed the maximum levels of IR-ANP to be attained 10 min after the infusion was stopped. The changes in plasma IR-ANP concentrations in response to isoproterenol were significantly reduced in rabbits given atenolol, a β_1-adrenoceptor-blocking drug. In nonvagotomized rabbits, smaller increases in plasma IR-ANP concentration were seen (Rankin et al., 1987b). In a later study, the effect of isoproterenol on mean right and left atrial pressures and dimensions was investigated in anesthetized rabbits (King et al., 1991). There was no significant change in mean right atrial pressure, left atrial pressure, or atrial dimension with the infusion of isoproterenol, showing that the release of IR-ANP in response to isoproterenol is not mediated by atrial stretch (King et al., 1991). However, an increase in heart rate following the administration of isoproterenol may contribute to the release of ANP; heart rate increased by 60 beats/min during the infusion of isoproterenol. β-Adrenergic stimulation by isoproterenol also produces slight increases in plasma NT-ANP associated with positive chronotropic and inotropic responses in conscious rats (Lachance and Garcia, 1991e).

Data obtained in vivo are not consistent, because a small decrease in plasma IR-ANP levels during administration of isoproterenol has been reported. When heart rate and right atrial pressure were kept constant during isoproterenol infusion (0.2 to 0.3 μg/kg/min) in pentobarbital-anesthetized open-chest dogs, plasma IR-ANP concentration decreased (Christensen et al., 1989b). The direct effect of β-adrenergic stimulation remains unclear, because the decrease in left atrial pressure may explain the reduction in plasma IR-ANP during isoproterenol infusion. In a recent study (Christensen et al., 1991), the effects of isoproterenol infused into the proximal part of the circumflex coronary artery on ANP release were studied in seven anesthetized pigs with open chests. During isoproterenol infusion, plasma IR-ANP concentration decreased by 20% from a control value of 76 pg/ml, suggesting that β-adrenergic stimulation inhibits ANP release. Heart rate and left atrial systolic pressure increased slightly in response to isoproterenol, whereas values for right atrial and mean aortic pressure were not shown. The difference between this and other in vivo studies is difficult to explain but may relate to the isoproterenol-induced hemodynamic changes, doses of isoproterenol used, and differences in experimental designs. Nevertheless, under these experimental conditions, calcium infusion and phenylephrine were found to increase plasma IR-ANP concentration.

In conclusion, the direct effects of α- and β-adrenergic stimulation on ANP release in vivo are generally smaller than the effect of an increase in atrial filling pressure. Hemodynamic effects of α- and β-adrenergic agonists on ANP release usually dominate in vivo. However, if increases in the heart rate, force of cardiac contraction, and total peripheral resistance induced by α- and β-adrenergic agonists are minimized in vivo as well as in vitro, a direct stimulatory effect on ANP release is revealed. Evidence also suggests that developed tension may be an important determinant of ANP release in response to certain adrenergic agonists, especially β-agonists both in vitro and in vivo. Under some experimental conditions, i.e., nonbeating atria (in vitro) or low atrial tension (in vivo), in the absence of a stimulatory effect of increased developed tension, the direct inhibitor effect of isoproterenol on ANP release may be revealed. Thus, it appears that beating atria in vivo respond like nonbeating atria in vitro if tension development is low during β-adrenergic stimulation.

3. Acetylcholine and cholinergic agonists. Isolated atria have been shown to release ANP-like bioactivity in response to acetylcholine; the increase in the bioactivity of

the incubation medium could be completely prevented by atropine (Sonnenberg et al., 1984). Similarly, atrial tissue fragments were shown to release IR-ANP into the incubation medium in a dose-dependent manner in response to acetylcholine (Naruse et al., 1986; Inoue et al., 1988a). In the isolated perfused rat heart preparation, infusion of 10^{-5} or 10^{-6} M acetylcholine resulted in a brief increase in hormone release, followed by a gradual decline (Ruskoaho et al., 1985; Toth et al., 1986). The increase in IR-ANP release was blocked by atropine,, suggesting that a muscarinic receptor was involved. Furthermore, carbachol (Matsubara et al., 1988a) or acetylcholine (Hayashi et al., 1988; Lew and Baertschi, 1989) stimulated IR-ANP secretion into the incubation medium of atrial cardiocytes cultured in serum-free media. In vivo carbachol increased plasma IR-ANP levels (Garcia et al., 1986a). Nicotine also increased plasma IR-ANP levels in conscious rabbits, and the stimulatory effect appeared to be mediated by peripheral nicotinic cholinergic mechanisms, because two ganglionic blockers, mecamylamine and trimethaphan, blocked the stimulation of ANP release by nicotine and adrenergic blockers had no effect (Larose et al., 1988).

In contrast, others have not been able to demonstrate IR-ANP release from atrial slices or superfused atria with cholinergic agonists (Lachance et al., 1986; Ferrari and Agnoletti, 1989). IR-ANP secretion was also not affected by superfusion of left atria with methacholine, which resulted in a marked decrease in developed tension (Schiebinger et al., 1987). Furthermore, methacholine markedly inhibited the norepinephrine- (Schiebinger et al., 1987), isoproterenol- (Schiebinger, 1988), and CGRP-stimulated (Schiebinger and Santora, 1989) IR-ANP secretion from rat atria; these effects of methacholine were blocked by atropine. Yet, methacholine did not inhibit the IR-ANP-releasing effect of phenylephrine (α-adrenergic effect) (Schiebinger, 1988). The contradictory results are difficult to explain, but differences in methodology may be important. Acetylcholine and cholinergic agonists also decrease heart rate and developed tension, which are known to influence ANP secretion if not kept constant (see above). Furthermore, muscarinic receptor agonists seem to alter potassium conductance, decrease calcium currents, activate guanylate cyclase, and stimulate the phosphoinositide pathway in target cells (Birnbaumer et al., 1990). Thus, it is likely that all these varied effects may account for the discrepant results.

4. Vasopressin. a. IN VITRO STUDIES. A number of reports demonstrate that ANP release can be stimulated by vasopressin both in vitro and in vivo. AVP has been shown to stimulate ANP release in vitro from atrial tissue fragments, whereas the nonpressor analog, deamino-8-D-arginine vasopressin, had no effect (Sonnenberg and Veress, 1984). Atrial tissue fragments were also shown to release IR-ANP into the incubation medium in response to AVP in a dose-dependent manner (Naruse et al., 1986). Incubation of atrial appendages in AVP-containing media resulted in significant release of natriuretic activity and proANP (Veress et al., 1988). Vasopressin at 100 nM increased IR-ANP release from cultured atrial myocytes into the incubation medium by 40 to 50% (Lew and Baertschi, 1989). AVP at concentrations of 10^{-5} to 2×10^{-5} M also increased IR-ANP release from isolated rat left atrium (Ishida et al., 1988). In addition, AVP and 1-deamino-AVP resulted in an increase of IR-ANP release from superfused atrial slices; a specific V_1-antagonist blocked the stimulatory effect of vasopressin on IR-ANP release, and a V_2-agonist, deamino-8-D-arginine vasopressin, had no effect (Zongazo et al., 1991). In contrast, Lachance et al. (1986) reported that vasopressin had no effect on IR-ANP release from minced atrial tissue. Furthermore, addition of vasopressin to the perfusate had no significant effect on IR-ANP release in a rat heart-lung preparation (Dietz, 1988). When atrial cardiocytes cultured in serum-free media were used, vasopressin failed to stimulate IR-ANP secretion into the incubation medium (Matsubara et al., 1988a). In isolated superfused rat left atria paced at 4 Hz, vasopressin also failed to influence basal and stretch-induced IR-ANP secretion (Schiebinger and Greening, 1992).

b. IN VIVO STUDIES. Pharmacological doses of vasopressin induce a marked, immediate increase in plasma IR-ANP levels in rats (Katsube et at., 1985; Manning et al., 1985; Katsube et al., 1986; Haass et al., 1987b; Itoh et al., 1987b; Jin et al., 1989), dogs (Inoue et al., 1988b; Lote et al., 1989; Zimmerman et al., 1990), rabbits (Wei et al., 1986; Larose et al., 1988; Courneya et al., 1989), and humans (Cases et al., 1992). In anesthetized rats, administration of vasopressin and 1-deamino-AVP (0.1 to 10 μg, i.v. bolus) produced a dose-dependent increase in plasma IR-ANP that peaked 5 to 15 min after the injection (Manning et al., 1985). The stimulated release of ANP was related to increased arterial pressure (Manning et al., 1985; Inoue et al., 1988b) or closely paralleled the elevations in atrial pressure (Katsube et al., 1985). Neither the nonpressor vasopressin analog (Manning et al., 1985; Inoue et al., 1988b) nor AVP in the presence of a specific pressor antagonist (Manning et al. 1985; Itoh et al., 1987b) caused IR-ANP to be released into the circulation. Oxytocin, which has <10% of the pressor activity of AVP, also increased plasma IR-ANP levels in rats (Manning et al., 1985).

There is also evidence that AVP may not act directly on the atria to release ANP in the intact animals. Salazar et al. (1986a) reported that an antagonist of the vasopressor action of AVP prevented the increase in blood pressure produced by an infusion of hypertonic saline in dogs but did not alter the ANP response; changes correlated well with the increase in the right atrial pressure. In conscious rats, the acute blockade of vasopressin by a vasopressin pressor antagonist did not block volume

expansion-stimulated plasma IR-ANP concentrations (Haass et al., 1987b). However, changes were not correlated with changes in atrial pressures or blood pressure. Ogawa et al. (1987) found that the dehydration-induced increase in AVP release per se did not enhance ANP release in rats, and plasma IR-ANP concentration decreased significantly during a rather substantial hemorrhage that certainly increased plasma AVP concentrations (Edwards et al., 1988c). Failure of hemorrhage to induce ANP release despite large increases in plasma AVP has also been reported by others (Geer et al., 1988; Shiraishi et al., 1990). Plasma IR-ANP levels did not change significantly in dehydrated and overhydrated conscious dogs in response to vasopressin infusions resulting in plasma AVP levels of 88 to 93 pg/ml (Hellebrekens et al., 1989). These data suggest that AVP does not directly stimulate ANP release when the intravascular volume is decreased by hemorrhage or dehydration. However, these findings do not exclude the possibility that V_1-receptor stimulation of ANP release was negated by the decrease in atrial filling pressure.

There is some evidence that changes in systemic arterial or atrial pressures may not be the only factors involved in the vasopressin-stimulated ANP release. Recent data show that vasopressin could be acting through a parallel but pressure-independent mechanism to augment ANP release. Itoh et al. (1987b) showed that a reduction of circulating blood volume (hemorrhage, 5 ml) in conscious rats attenuated the action of AVP on IR-ANP secretion, suggesting that the effect of AVP is modulated by the intravascular volume, possibly via atrial stretch. Furthermore, ANP secretion induced by volume loading (saline, 3 ml) was significantly enhanced by the coadministration of 5 ng of vasopressin, suggesting a modulatory role for AVP in the control of ANP secretion (Itoh et al., 1987b). However, because atrial pressures or other hemodynamic parameters were not measured, the role of vasopressin in the control of ANP secretion remained unclear.

In the pithed rat, vasopressin infusion restored the heart's ability to release ANP in response to acute volume expansion (Ruskoaho et al., 1989c). In that study, the effects of humoral factors (vasopressin, ANG, epinephrine) and the stimulation of parasympathetic and sympathetic nerves on basal and atrial stretch-induced ANP release were studied. In response to volume expansion, the IR-ANP versus right atrial pressure curve shifted to the right, showing that much smaller amounts of IR-ANP were released for a given increase in right atrial pressure in pithed than in conscious rats (Ruskoaho et al., 1989c). When AVP was infused into pithed rats, mean arterial pressure increased, but basal plasma IR-ANP concentration did not change significantly. However, acute volume expansion in the presence of vasopressin infusion increased the amount of circulating IR-ANP by a factor of 4, as observed in normal conscious rats. When AVP infusion was discontinued, the restoration of volume expansion-induced ANP release was markedly attenuated and the V_1-antagonist blocked the increase in mean arterial pressure as well as the increase of plasma IR-ANP concentration produced by AVP (Ruskoaho et al., 1989c). In contrast, a V_2-receptor agonist had no effect on hemodynamics or on plasma IR-ANP concentrations in response to volume expansion. Exogenous vasopressin infusion also restored the volume expansion-stimulated ANP release in hypophysectomized animals (Arjamaa et al., 1988; Ruskoaho et al., 1989c). The ability of vasopressin to restore the volume expansion-stimulated ANP release in pithed and hypophysectomized rats by stimulating V_1-receptors shows the synergistic effect of atrial pressure and vasopressin in the regulation of ANP release.

Vasopressin has also been shown to enhance atrial stretch-induced release of ANP in conscious vasopressin-deficient Brattleboro and Long-Evans rats (Ruskoaho et al., 1989b). Infusion of vasopressin alone in a dose of 5 ng/kg/min for 30 min had no effect on right atrial pressure and plasma IR-ANP levels in either strain but slightly increased mean arterial pressure in Long-Evans rats. Yet, in both Brattleboro and control rats vasopressin enhanced the amount of plasma IR-ANP released in response to volume load; the relation between the change in plasma IR-ANP concentration and the change in right atrial pressure shifted to the left. The greater amount of IR-ANP released in response to acute volume expansion in the vasopressin-treated rats than in the control rats further supports the concept that the ANP release in response to stretching is hormonally modulated. However, baseline plasma levels of IR-ANP were not different in control animals and Brattleboro rats, which totally lack vasopressin and acute volume expansion with saline for a given increase in right atrial pressure caused a similar marked increase in plasma IR-ANP levels in both strains (Ruskoaho et al., 1989b), showing that vasopressin is not essential for the ANP secretory response to acute volume expansion. These results differ from those of Sakata et al. (1988b) in Brattleboro rats. They found that volume expansion (30 to 40% of the normal blood volume) in conscious DI rats did not stimulate plasma IR-ANP release but caused a marked ANP release into the plasma of anesthetized rats. Pesonen et al. (1990) found an attenuated ANP secretory response to fentanyl, a μ-receptor agonist, in conscious Brattleboro rats compared to that in normal Long-Evans rats, suggesting that vasopressin may modulate the opiate-stimulated plasma IR-ANP release. Furthermore, V_1-antagonists have been shown to decrease plasma ANP-levels induced by central osmotic stimulation (Iitake et al., 1989) or ET (Yamamoto et al., 1991).

In conclusion, the release of ANP in intact animals can be induced by a naturally occurring peptide hormone, AVP. ANP, in turn, suppresses vasopressin release,

showing the existence of a negative-feedback endocrine loop. Experimental evidence suggests that vasopressin, as well as probably other pressor hormones (see section IV.E.1), elevates plasma ANP levels by two different mechanisms: (*a*) a direct increase in atrial distension, because changes in plasma IR-ANP concentration correlate with changes in atrial pressure or dimensions, and (*b*) possible enhancement of the stretch-mediated ANP release (fig. 4). The question arises whether vasopressin-mediated ANP release might play a regulatory role in vivo. Normally, high vasopressin states are associated with antinatriuresis rather than natriuresis. A possible explanation is that ANP in these situations may oppose the renal, hemodynamic, and hormonal effects of vasopressin. The stimulatory action of vasopressin on atrial stretch-induced release of ANP might be significant during rapid dehydration that has been associated with rapid changes in plasma IR-ANP and vasopressin (Januszewicz et al., 1986a,b; Edwards et al., 1988c). Furthermore, the high concentrations of pressor hormones in heart failure, which contribute to the vasoconstriction in that disease, may be related to increased plasma ANP levels observed in patients by facilitating atrial stretch-induced ANP secretion from the heart. Nevertheless, it is evident that regulation of ANP release involves a complex interaction between stretch and humoral factors, which must be taken into consideration when determining the physiological and pathophysiological role of ANP. The intracellular mechanism for this new mechanism by which humoral stimulation appears to modulate the direct, mechanical stimulus-induced hormone secretion will be discussed in detail later.

5. Angiotensin. a. IN VITRO STUDIES. A possible direct effect of ANG II on ANP release is suggested by in vitro studies, although studies performed in isolated organ preparations have given conflicting results. Incubation of atrial appendages in ANG-containing media resulted in significant release of natriuretic activity and immunoreactive proANP (Veress et al., 1988; Sonnenberg et al., 1989); when the atria were not gently scraped (to remove most of the endocardium) before experiments, ANG failed to increase immunoreactive proANP release into the incubation medium (Sonnenberg et al., 1989). In contrast, addition of ANG into the perfusate had no significant effect on IR-ANP release in a rat heart-lung preparation (Dietz, 1988). Results of two other studies tend to support this finding, because the in vitro incubation of atria with ANG II did not stimulate IR-ANP secretion into the incubation medium (Naruse et al., 1986; Lachance and Garcia, 1988). Using atrial cardiocytes cultured in serum-free media ANG failed to stimulate IR-ANP secretion into the incubation medium (Matsubara et al., 1988a; Glembotski et al., 1991).

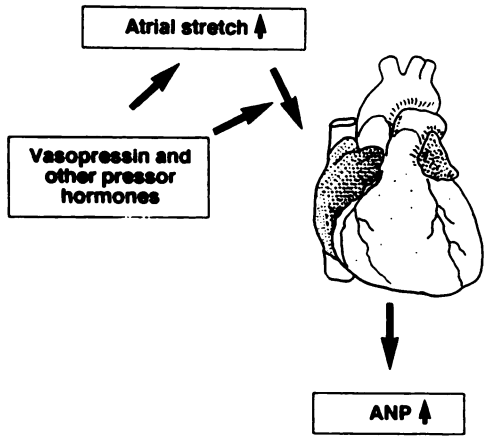

FIG. 4. Mechanisms of the effect of pressor hormones on ANP secretion.

b. IN VIVO STUDIES. The literature on the actions of ANG II on ANP release is somewhat contradictory, and it is not yet clear whether its effect in vivo is secondary to the hemodynamic changes (resulting in increased atrial stretch) or due to direct receptor stimulation. Large pressor doses of ANG II have been shown to promote IR-ANP release in vivo in rats (Katsube et al., 1985; Manning et al., 1985; Haass et al., 1987b; Lachance and Garcia, 1988, 1989), dogs (Edwards et al., 1986b; Stewart et al., 1990), and humans (Uehlinger et al., 1986; Tunny et al., 1987; Didden et al., 1991). Some of these findings suggested that ANG II is a more potent stimulator of ANP release than other agonists, such as phenylephrine and norepinephrine. The ANG II-induced ANP release has been ascribed to hemodynamic effects of ANG II leading to increased atrial stretch. In particular, the results in conscious dogs by Stewart et al. (1990) in which both atrial diameters and atrial pressures and, therefore, atrial stretch could be measured support the concept that ANG II-induced ANP release depends on the amount of atrial stretch; for an equal increase in left atrial stretch caused by aortic constriction or by ANG II infusion, similar increases in plasma IR-ANP levels were found. Examinations in open-chest dogs (Christensen et al., 1989b) and in conscious rats (Lachance and Garcia, 1989) also showed that the increase in left atrial pressure could explain the high plasma IR-ANP level during infusion of ANG II.

However, results of some in vivo studies suggest that the increase in plasma IR-ANP concentration induced by ANG II is mediated by mechanisms other than its systemic hemodynamic actions. Ruskoaho et al. (1989c) studied the effect of 30-min infusions of ANG II on basal and volume expansion-induced plasma IR-ANP levels in pithed rats. ANG II infusion and volume expansion alone had no significant effect on plasma IR-ANP levels, whereas volume expansion in the presence of ANG induced significant increases in plasma IR-ANP concentration, showing that the release of ANP from the heart in response to atrial distension appears to be modulated by ANG. The stimulatory response to volume expansion in the presence of ANG II was smaller to that in the presence of vasopressin in spite of the fact that equi-

pressor doses of both were used (Ruskoaho et al., 1989c). In volume-repleted (sustained volume expansion and converting enzyme inhibition) anesthetized or conscious dogs, the doses of ANG II (2.5 to 10 ng/kg/min i.v.), which should result in circulating levels within the physiological range, were able to increase plasma IR-ANP concentration without changing atrial pressures, heart rate, or hematocrit (Volpe et al., 1990). Although blood pressure increased, the increase in ANP did not correlate with the associated changes in systemic blood pressure during ANG II infusion (Volpe et al., 1990). Furthermore, in anesthetized or conscious rabbits, ANG II infusion (for 30 min) caused a marked increase in plasma IR-ANP levels even when the effect of ANG II on systemic and right atrial pressures was prevented by the concomitant administration of nitroprusside (Volpe et al., 1991b). Yet, left atrial pressure was not measured in that study. It is also possible that the stimulatory effect of ANG II on ANP release is amplified by volume expansion. In fact, it has been reported that i.c.v. administration of ANG II enhances IR-ANP secretion induced by volume loading (Itoh et al. 1988b) but does not affect basal plasma IR-ANP levels (Itoh et al. 1988b). This finding may have particular relevance to pathological states in which increased levels of ANG II and ANP coexist.

Thus, under certain experimental conditions, stimulation of ANP secretion could not be explained by hemodynamic responses to ANG II, although it cannot be completely ruled out that the increase in systemic blood pressure plays a part, especially at high doses of ANG II. These results suggest that ANG II may exert a significant modulatory effect on ANP secretion. The mechanisms by which ANG II may promote ANP release independently of its hemodynamic actions are unclear. A direct action of ANG II may depend on increased tension in the myocyte, because ANG II increases inward calcium fluxes and force of contraction in atrial myocytes. Experiments on atrial cell cultures are required to identify the mechanisms.

E. Endothelium-derived Contracting and Relaxing Factors

One of the greatest growth areas in cardiovascular science in recent years has been the study of the role of the endothelium. Endothelium is recognized as a cardiovascular endocrine organ in its own right, occupying a critically strategic interface between blood and body and subserving a multitude of regulatory roles (for reviews, see Furchgott and Vanhoutte, 1989; Katusic and Shepherd, 1991). Endothelial cells line the inner surface of blood vessels, including atrial blood vessels and the much-trabeculated cavity of the cardiac chambers. Endothelial cells sense the transmural pressure at the endothelial surface, and release of endothelium-dependent vasoconstrictor and relaxant factors has been observed in response to mechanical stretch and increase in transmural pressure. Furthermore, bovine aortic cells, when placed in coculture with rat atrial myocytes, stimulate the release of ANP (Lew and Baertschi, 1989). Of the factors released from endothelial cells, ET, EDRF, and PGs have been reported to influence ANP secretion from cardiocytes.

1. Endothelins. ETs are a family of regulatory peptides originally identified in cultured porcine endothelial cells (for reviews, see Yanagisawa and Masaki, 1989; Masaki et al., 1991; Rubanyi and Botelho, 1991; Simonson and Dunn, 1991). ET appears to have a short $t_{1/2}$ in plasma, and the majority of circulating ET is cleared after one pass through the lungs, showing that ET functions as a local hormone synthesized by specific endothelial or epithelial cells and acts in either a paracrine or autocrine mode on target cells. However, ET-like immunoreactivity has been detected in plasma, suggesting that it may also be a circulating vasoconstrictor in humans.

Soon after the discovery of ET-1, it became apparent that in vascular preparations the ETs were the most potent vasoconstrictor substances yet identified. The vasoconstrictor actions of ET-1 in vivo are opposed by the concomitant release of ANP in dogs (Goetz et al., 1988; Miller et al., 1988; Tsuchiya et al., 1990; Donckier et al., 1991). In conscious (Goetz et al., 1988) and anesthetized (Miller et al., 1988) dogs, the progressive increase in plasma IR-ANP concentration during ET infusion corresponds to the progressive increase in atrial pressure, also observed with ET. This supports previous reports of the mechanism of ANP release through elevation in atrial pressure in response to infusion of pressor agents. Administration of ET also produces a profound increase in plasma IR-ANP levels in anesthetized and conscious rats (Stasch et al., 1989b; Garcia et al., 1990b; Kohno et al., 1990a; Öhman et al., 1990). ET-1 given i.c.v. also increases plasma IR-ANP concentration in rats (Yamamoto et al., 1991).

Pharmacological studies in vitro show that ET also directly increases ANP secretion from the heart and not solely via the hemodynamic effects of ET. ET has been shown to be a potent ANP secretagogue in cultured rat atrial myocytes (Fukuda et al., 1988, 1989; Lew and Baertschi, 1989; Sei and Glembotski, 1990; Gardner et al., 1991; Uusimaa et al., 1992a), isolated rat atria (Hu et al., 1988a; Stasch et al., 1989b; Winquist et al., 1989; Schiebinger and Gomez-Sanchez, 1990; de Bold et al., 1991; Schiebinger and Greening, 1992), and isolated perfused rat heart (Mäntymaa et al., 1990; Pitkänen et al., 1991). In the isolated perfused rat heart preparation, at the doses at which vasoconstrictor and cardiac effects (0.36 to 2.7 nmol/l) were observed, ET increased IR-ANP concentration in the perfusate (Mäntymaa et al., 1990). In this respect, ET was 10- to 100-fold more potent than phorbol esters or other agents previously shown to induce ANP release. Under these experimental condi-

tions, ET is the most potent ANP secretagogue, on a molar basis, yet identified in the perfused rat heart. Sarafotoxin-b, the most potent ET peptide from the venom of the snake *Atractaspis engaddensis* also stimulated IR-ANP secretion dose dependently in the perfused rat heart preparation with a potency similar to that of ET-1 (Pitkänen et al., 1991). Maximal ANP secretion was not sustained at higher (100 nM) ET concentrations (Sei and Glembotski, 1990; Schiebinger and Gomez-Sanchez, 1990). When isolated atrial cells were studied, the rate of ANP secretion was initially high (maximum 10 min) and declined after 30 min to a lower value which was significantly greater than the basal secretion rate (Sei and Glembotski, 1990).

The function of ET as a physiological regulator of ANP secretion remains to be established. It is notable that the expression of the ET gene is regulated by several vasoactive agents such as thrombin and epinephrine (Masaki et al., 1991), which are known to stimulate phosphoinositide hydrolysis in endothelial cells. ANG, vasopressin, calcium ionophore A23187, endotoxin, and hypoxia are also known to stimulate gene expression and release of ET. Physical factors, such as chronic low-level fluid mechanical shear stress, increased both the mRNA for proET and supernatant ET recovery in cultured endothelial cells (Masaki et al., 1991). Because atrial stretch and pressor hormones increase ANP release, these observations raise the interesting possibility that ET may be involved in mediating the atrial stretch-induced ANP release as well as the effects of pressor hormones on the stretch-activated release of ANP.

To test this hypothesis, Mäntymaa et al. (1990) used a modification of the perfused heart preparation that permitted distension of the right atrium. A mean increase in right atrial pressure of 2 mm Hg produced by elevation of the pulmonary artery cannula tip resulted in a 52% increase in the rate of IR-ANP release into the perfusate. ET dose dependently augmented the stretch-stimulated ANP release, by shifting the ANP versus right atrial pressure curve to the left. Thus, for a given increase in right atrial pressure in the presence of ET, more ANP was released in the isolated perfused heart preparation (Mäntymaa et al.. 1990). In support of this, Gardner et al. (1991) reported that in neonatal atrial cell cultures the response to ET administration in the presence of cyclical stretch was significantly greater than either cyclical stretch or ET alone. ET enhanced the ANP secretory response to stretch by 33% in the superfused rat left atria preparation (Schiebinger and Greening, 1992). These studies suggest an interaction between ET and mechanical stretch in controlling ANP secretion.

Evidence indicates that ET affects circulatory hemodynamics and the response to alterations in cardiorenal homeostasis in pathological states in the human, including coronary spasm and hypertension. The observation that ET can release ANP from the heart has been extended by Winquist and associates (1989), who assessed the ability of ET to release ANP from isolated, spontaneously contracting atria obtained from hypertensive and normal rats. In that study, ET was noted to be a potent secretagogue for the release of IR-ANP from atria in both groups, but the amount released from the hypertensive rat atria was greater with an equal dose of ET. In contrast, in a study by Mäntymaa et al. (1990), ET failed to cause IR-ANP release from SHR hearts. In the isolated perfused heart preparation, the vasoconstrictor response to ET infusion was greater in SHR than in normotensive WKY rats, and thus a lower dose of ET was used to achieve equipotent doses and identical contractile and vascular effects in both strains. The reason for these discrepant results is not clear, but the differing effect of ET on ANP release between SHR and WKY rats suggests that hypertension alters intracellular regulatory mechanisms and that ET may play a role in the pathogenesis of vascular diseases such as hypertension.

In conclusion, ET has both paracrine and endocrine characteristics, which, when released by the endothelial cells, will affect ANP release. The fact that vascular endothelial cells produce a substance that can augment atrial stretch-induced ANP release shows that the endothelium may be involved in the regulation of atrial stretch-mediated hormone release (fig. 5). Certainly, more data concerning the role of the endothelium in modulating ANP release from the heart is needed. However, it appears that the endothelium may act as a sensor for the regulation of ANP release.

2. Endothelium-derived relaxing factor. In addition to ET, endothelial cells release a factor that relaxes blood vessels named EDRF by Furchgott and Vanhoutte (1989). EDRF possesses chemical and pharmacological characteristics that are identical with those of nitric oxide (for reviews, see Furchott and Vanhoutte, 1989; Katusic and Shepherd, 1991). One important physiological stimulant of EDRF release is flow rate acting through the relatively small longitudinal shear force experienced uniquely by the endothelium. In support of the hypothesis that EDRF might be involved in the regulation of ANP release, coculture of bovine aortic endothelial cells with neonatal rat atrial myocytes induced a 2.1-fold increase in IR-ANP release in the medium, and this release could be inhibited by 47% with 10 μM acetylcholine, which is known to evoke the release of EDRF (Lew and Baertschi, 1989). Sanchez-Ferrer et al. (1990) studied the effect of inhibitors of EDRF on the release of ANP from isolated rat atria. Saponin treatment, which effectively removes endocardium, caused a marked increase in IR-ANP release into the perfusate. Methylene blue, oxyhemoglobin, or hydroquinone, agents that are known to inhibit the release of EDRF by different mechanisms, caused an increase in the basal release of IR-ANP (Sanchez-Ferrer et al., 1990). These results suggest that the release of EDRF from either endocardial cells

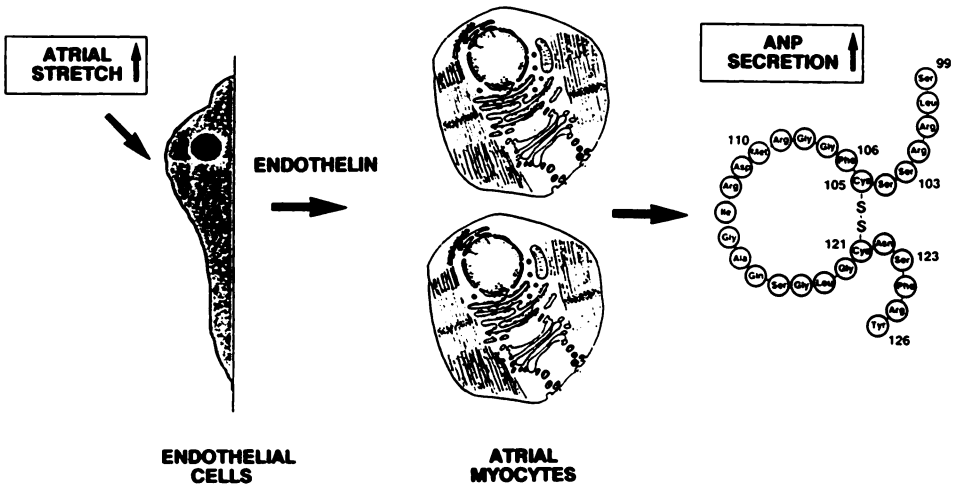

FIG. 5. Possible role of ET in the regulation of stretch-induced ANP secretion.

or endothelial cells of the atrial blood vessels may exert a tonic inhibitor effect on the ANP secretion from atria. However, in another study, methylene blue and hydroquinone did not affect basal or vasopressin stimulated release of IR-ANP from atrial slices (Zongazo et al., 1991). The physiological importance of these observations remains to be established.

3. Arachidonic acid and its metabolites. PGs may also have a role in enhancing basal and stretch-induced release of ANP. PGs are found in significant levels in heart tissue, and stretch has been shown to increase PG release in a number of other systems. PGs, specifically $PGF_{2\alpha}$ and PGE_2, stimulate ANP secretion in cultured neonatal atrial and ventricular cells and in vivo (Gardner and Schulz, 1990; Kovacic-Milivojevic et al., 1991). $PGF_{2\alpha}$ infusion in anesthetized rats within 2 min of administration resulted in a significant increase in plasma IR-ANP levels. Furthermore, pretreatment of rats with indomethacin resulted in about a 50% reduction in the increment of plasma IR-ANP levels 2 and 5 min after an 8-ml volume infusion. Treatment with PG (10^{-7} to 10^{-5} M) resulted in stimulation of IR-ANP secretion from cultured neonatal myocytes at 24 h, probably caused by an increase in ANP biosynthesis as reflected by elevated ANP mRNA levels (Gardner and Schulz, 1990). In separate experiments, atrial cells treated with $PGF_{2\alpha}$ (10^{-5} M) for 5 to 15 min significantly increased IR-ANP release into the incubation medium (Gardner and Schulz, 1990; Kovacic-Milivojevic et al., 1991), suggesting a direct stimulatory effect of PGs on ANP release from atrial myocytes. Interestingly, indomethacin blocked AVP-induced ANP release from isolated atrial slices (Zongazo et al., 1991) but had no effect on the basal IR-ANP secretion from isolated rat atria (Sanchez-Ferrer et al., 1990) and atrial slices (Zongazo et al., 1991).

Of the other products of arachidonate metabolism tested, leukotriene C_4 showed a modest but dose-dependent increase in IR-ANP release from atrial myocytes after 24 h of treatment but no effect after 30 min of treatment (Kovacic-Milivojevic et al., 1991). However, in anesthetized rats nordihydroguarietic acid, a lipoxygenase inhibitor, given 30 min prior to volume expansion, had no effect on stretch-dependent release of ANP. These studies suggest that the eicosanoids, especially PGs, may have an important local role in the physiological regulation of both basal and stretch-induced ANP secretion as well as in pathophysiological states characterized by PG overproduction. The results also support the concept that local paracrine factors released from endothelial cells may modulate ANP release from the heart.

F. Role of the Peripheral and Central Nervous System

Despite a large number of studies, the role of the central and peripheral nervous system in ANP secretion is still unclear. The heart is richly innervated by adrenergic and cholinergic fibers. The atria contain numerous unencapsulated nerve endings which, histochemically, resemble arterial baroreceptors and are believed to monitor the blood volume. Distension of the atria activates these local stretch receptors with afferent fibers in the vagus nerves. Increased afferent nerve activity then initiates a central reflex, resulting in a decrease in the cardiac vagal efferent activity and an increase in discharge of cardiac sympathetic efferent nerves (for a recent review, see Hainsworth, 1991). Several studies have also demonstrated that stimulation of cardiopulmonary receptors by either atrial stretch or volume expansion induces diuretic and natriuretic responses that are diminished by vagotomy (Linden, 1979). Nerve impulses arising from stretch receptors, traveling via the vagus nerves to the brain, may also alter secretion of several vasoactive humoral substances such as vasopressin and ANG. The findings that neurotransmitters released at the nerve terminals of the autonomic nervous system, noradrenaline and acetylcholine (see section IV.D), and several other humoral substances affect the ANP secretion rate also suggest that cardiac vagal affer-

ent nerves or cardiac sympathetic efferent nerves may be involved in the regulation of ANP secretion. Finally, the fact that ANP release occurs in vitro in response to atrial stretch in isolated perfused hearts, isolated atria, and isolated cells in culture does not exclude the possibility of the modulatory action of specific brain regions and the central and peripheral nervous system on ANP release.

1. Pithed rats. The experiments in a pithed rat preparation provided the first evidence that neuronal influences may be important in the regulation of ANP secretion. First, Pettersson et al. (1985) reported that pithing, which removes both humoral influences originating from the central nervous system and direct neural control of the heart via the vagal and sympathetic nerves, decreased baseline plasma IR-ANP levels in rats. Eskay et al. (1986) then showed that pithing blocked the volume load-induced increase in plasma IR-ANP concentration. Other studies have confirmed that highly significant elevations in right atrial pressure in response to volume loading are incapable of eliciting the ANP release response in pithed rats (Haass et al., 1987b; Zamir et al., 1987; Ruskoaho et al., 1989c). In addition, electrical vagal or sympathetic nerve stimulations, changes in heart rate, and infusion of pressor doses of vasopressin, ANG, or epinephrine for 30 min failed to increase plasma IR-ANP levels in pithed rats (Ruskoaho et al., 1989c). In another study, the plasma IR-ANP in the pithed rat did not reach the values found in intact rats even at the high concentrations of vasopressin and norepinephrine infused (Haass et al., 1987b). These results obtained with the denervated heart preparation (pithed rat) suggest that neuronal and/or humoral influences are important in the volume loading-induced release of ANP from the heart. However, the effects of pithing are complex and include changes in effective blood volume and cardiac filling pressures. Therefore, the blockade of volume expansion-induced ANP release is most likely to be explained by hemodynamic factors affecting the response of atrial distension to infusion of volume load or other stimuli. In addition, pithing may affect the production of humoral factors that either stimulate the release of ANP or alter atrial responses to stretch. However, no evidence for brain "ANP-releasing or inhibiting factor(s)" other than pituitary vasopressin was found in the experiments in pithed rats (Ruskoaho et al., 1989c).

2. Cardiac denervation and stimulation of sympathetic and parasympathetic nerves. Little or no influence of the ANP response to volume loading has been observed after vagotomy or cardiac denervation. Ledsome et al. (1985, 1986) reported that in dogs the increase in plasma IR-ANP concentration 20 min after mitral obstruction was not affected by a combination of bilateral cervical vagotomy and cardiac β-adrenoceptor blockade. A pronounced increase in plasma IR-ANP concentration measured in rats during water immersion was not prevented by bilateral transection of the vagi (Katsube et al., 1985) and showed the same response as controls to volume loading (Zamir et al., 1987), suggesting that the parasympathetic nervous system does not modulate the ANP secretory response to acute volume expansion in vivo. Moreover, acute parasympathetic nervous system and ganglionic blockade do not affect basal or volume-induced release of IR-ANP in rats (Haass et al., 1987b), and vagotomy had no effect on the IR-ANP release induced by volume expansion in in situ perfused rat hearts (Kabayama et al., 1987).

The results obtained after cardiac denervation or nerve stimulations are, in general, consistent with those reported after vagotomy. Goetz et al. (1986) showed that cardiac denervation did not affect the increase in plasma IR-ANP levels 30 min after atrial ballooning in conscious dogs. Similar increases in plasma IR-ANP in response to volume load was also noted in control and cardiac denervated dogs (Williams et al., 1990). Nishida et al. (1987) infused isoosmotic and isooncotic dextran-saline in conscious intact and cardiac denervated dogs. The baseline plasma IR-ANP levels did not differ between control and denervated animals, and in response to the increase in mean left atrial pressure, plasma IR-ANP levels (unextracted) increased about 2.2-fold in normal dogs and 2.4-fold in denervated dogs (Nishida et al., 1987, 1988a). The slope of the regression line comparing the changes in plasma IR-ANP concentration and mean left atrial pressure was also similar in both groups. Similarly, pharmacological blockade of cardiac nerves with intrapericardial procaine HCl did not alter resting plasma IR-ANP levels and also had no effect on the ANP response to blood volume expansion in conscious rabbits (McGrath et al., 1987). In anesthetized vagotomized rabbits, efferent stimulation of the right vagus nerve or right inferior cervical ganglion in the dog (Rankin et al., 1986) and the rabbit (Rankin et al., 1987b) did not produce significant changes in plasma IR-ANP concentrations. However, insufficient activation of cardiac β-adrenoceptors (heart rate increased by 34 beats/min) could explain why sympathetic stimulation did not evoke ANP release, because a high level of cardiac activation is necessary for tachycardia to stimulate ANP release in vivo. In pithed rats, the parasympathetic and sympathetic nerve stimulations that produced the expected hemodynamic alterations did not affect the volume expansion-stimulated plasma IR-ANP levels (Ruskoaho et al., 1989c). These findings suggest that the cardiac nerves may not affect the baseline plasma ANP levels or modulate the increase in ANP release enhanced by atrial distension.

Results of some experimental studies and observations, however, suggest that cardiac nerves, notably vagal pathways, may affect the regulation of ANP release. In conscious rats, sinoaortic denervation suppressed the response of plasma IR-ANP concentration to hypertonic saline (2.6- versus 5.5-fold increase in the sham-operated

animals) (Morris and Alexander, 1988). Baroreceptor denervation also produced significant decreases in basal IR-ANP levels. However, hematocrit decreased slightly less (4 versus 8% in the control group) in response to hypertonic saline, and atrial pressure was not measured simultaneously with ANP secretion (Morris and Alexander, 1988). Bilateral vagotomy appeared to increase basal plasma IR-ANP levels (Courneya et al., 1989; Phillips et al., 1989; Skepper et al., 1989) and to inhibit the expected increase of hypothalamic tissue IR-ANP concentration accompanying hypotensive hemorrhage (Phillips et al., 1989). In a study of conscious dogs (Pichet et al., 1988), plasma IR-ANP concentration in response to volume expansion did not increase more in cardiac denervated dogs despite significantly greater increases in left atrial and right atrial pressures. However, mean arterial blood pressure increased less in denervated dogs, and atrial dimensions were not measured. Furthermore, in anesthetized vagotomized rabbits, vagal stimulation produced a significant increase in left and right atrial pressures; yet, no increase in plasma IR-ANP concentration was observed (Rankin et al., 1987b), suggesting that some component of vagal stimulation suppressed the release of ANP that one would have expected to accompany the increase in atrial pressure. In addition, in anesthetized vagotomized rabbits (aortic depressor nerves also sectioned), plasma IR-ANP concentration was significantly greater when carotid sinus pressure was kept at 60 mm Hg compared to when it was 160 mm Hg, suggesting that baroreceptors may regulate ANP release (King et al. 1989b). Hexamethonium pretreatment abolished the ANP response, and thus, the inverse relationship between carotid sinus pressure and plasma IR-ANP appears to be mediated by the associated hemodynamic changes (King et al., 1989b). Finally, external neck pressure in hypertensive patients was found to increase blood pressure and forearm vascular resistance and decrease plasma IR-ANP concentration (Volpe et al., 1988a).

3. Pituitary, hypothalamus, and other areas in the central nervous system. More evidence for the role of the central nervous system in the regulation of plasma IR-ANP concentration was provided by observations that hypophysectomy in rats lowers basal plasma IR-ANP levels (Arjamaa et al., 1988) and blocks the increase in plasma IR-ANP concentration induced by volume expansion (Zamir et al., 1987; Arjamaa et al., 1988; Dietz and Nazian, 1988; Ruskoaho et al., 1989c) and stress (Blizard and Morris, 1987). The change in the right atrial pressure was similar in control and hypophysectomized rats in response to volume expansion (Arjamaa et al., 1988). In an isolated heart-lung preparation, hypophysectomized rats, which had decreased atrial IR-ANP concentrations, secreted significantly less IR-ANP than did intact rats during basal and stimulated conditions (Dietz and Nazian, 1988). Similarly, hypophysectomy decreased circulating IR-ANP levels as well as the NaCl-stimulated release and the basal secretion rate of IR-ANP from isolated paced rat atria in vitro (Arjamaa, 1989). These results suggest that hypophysectomy leads to an attenuation of the ANP response to blood volume expansion and that the intact pituitary is required to maintain normal ANP secretion. In support of this, the infusion of posterior lobe extracts enhanced volume expansion-stimulated plasma IR-ANP levels in pithed rats, whereas extracts of anterior pituitary, brain cortex, or hypothalamus had no effect (Ruskoaho et al., 1989c). Because infusion of V_1-antagonist blocked the ANP secretory response produced by infusion of posterior pituitary extract, AVP appears to be the factor in posterior pituitary extracts that restored the atrial stretch-induced release of ANP in the pithed rats.

However, the effects of hypophysectomy are complex and include changes in several hormones. Hypophysectomized rats also showed decreases in mean arterial pressure and heart rate (Zamir et al., 1987; Arjamaa et al., 1988; Dietz and Nazian, 1988), right atrial pressure, and blood volume, all of which attenuate the ANP response to volume expansion. Furthermore, the lower atrial IR-ANP content (synthesis) as a result of the lower metabolic state of the hypophysectomized rats may be the primary mechanism for the lower IR-ANP secretion rate observed in vitro (Dietz and Nazian, 1988), although the concentrations of IR-ANP in the left and right atria have been shown to be normal or higher than that in sham-operated animals (Zamir et al., 1987; Arjamaa et al., 1988). Hypophysectomy (4 weeks) also had no effects on ANP mRNA levels in atrial cells (Arjamaa et al., 1990). Dexamethasone substitution treatment of hypophysectomized rats significantly increased the secretory response to a hyperosmotic NaCl perfusion in isolated atria (Arjamaa et al., 1990), suggesting that glucocorticoids may be involved in the attenuated release after hypophysectomy.

Zamir et al. (1987) provided evidence that suggests a role for the anterior pituitary in the modulation of ANP secretion. They found that basal and blood volume expansion-stimulated increases in plasma IR-ANP concentration were significantly blunted in hypophysectomized rats (8 days after operation) but were restored (10 days after operation) when the resected pituitary or anterior pituitary was reimplanted under the kidney capsule. No significant effect on ANP release in response to acute volume expansion was observed in hypophysectomized rats bearing a neurointermediate lobe autotransplant (Zamir et al., 1987). Acute volume expansion-induced IR-ANP release was also blunted in rats with carotid artery and jugular vein occlusions, suggesting a hormonal mechanism in ANP release (Zamir et al., 1987). Thus, hormones derived from the pituitary gland may exert their effects directly in myocytes to facilitate ANP release or indirectly through an action on other endocrine organs such as the thyroid and adrenal glands.

A central nervous system region that is critical in the regulation of sodium metabolism is the AV3V. Lesions in this region alter renal secretion of sodium and prevent a number of volume-dependent forms of hypertension. There is also evidence that ANP-containing neurons are present in this region and send projections to the paraventricular nucleus (Standaert and Saper, 1988). There is some evidence that the AV3V region influences plasma IR-ANP concentration. Injections of carbachol (0.1 μg, i.c.v.) produced a 15-fold increase in plasma IR-ANP concentration within 20 min in rats with a chronically indwelling third-ventricle cannula (Baldissera et al., 1989). This central effect of carbachol appeared to be mediated by M_1-receptors based on the use of four selelective muscarinic receptor antagonists (Massi et al., 1991). Conversely, lesions of the AV3V region decreased plasma IR-ANP concentration 8-fold within 24 h, and this decrease was maintained for 120 h. Furthermore, in a study by Rauch et al. (1990), the effect of AV3V lesions on basal, volume-stimulated, and osmotic-stimulated plasma IR-ANP levels was determined in conscious rats 4 weeks after operation. Under basal conditions, there were no differences in plasma IR-ANP concentration between sham- and AV3V-lesioned rats. Infusion of either hypertonic saline or a continuous or bolus infusion of saline increased plasma IR-ANP concentration in sham-operated rats, whereas lesions in the AV3V region inhibited both volume- and osmotic-induced increases in plasma IR-ANP concentration. In agreement with this finding, Antunes-Rodrigues et al. (1991) reported that the IR-ANP response to volume expansion was significantly less in rats with AV3V lesions (studied 3 days after operation) than that in sham-operated controls, suggesting that the AV3V region may be involved in the regulation of plasma ANP concentration. Median eminence lesions also almost completely blocked the response to volume loading at 24 and 120 h after operation (Antunes-Rodrigues et al., 1990).

However, it is not possible to determine from these data whether lesions of the AV3V region directly reduce the ability of the heart (or brain) to release ANP or alter plasma IR-ANP concentration indirectly by affecting hemodynamic conditions or basal blood volume. In fact, the AV3V lesions were accompanied by marked hypernatremia (Rauch et al., 1990; Antunes-Rodrigues et al., 1991), suggesting that changes in blood volume, and thus in atrial distension, may have altered the responses to volume expansion. The same may be true for other compounds, such as clonidine (Pan and Gutkowska, 1988) and saline, which have been reported to increase plasma IR-ANP levels after central injection. In one study, an increased Na^+ concentration in cerebral spinal fluid in conscious rats increased plasma IR-ANP levels as well as blood pressure (Kawano et al., 1988), suggesting that sodium stimulus on the central nervous system may regulate ANP release from the heart. The combination of hexamethonium and vasopressin antagonist also inhibited the ANP secretory response to infusion of high sodium cerebral spinal fluid. Yet, the role of central osmoreceptors in the regulation of ANP release is not clear, because hypertonic sodium chloride injection i.c.v. had no affect on plasma IR-ANP levels in other studies (Hansell et al., 1987; Hattori et al., 1988; Iitake et al., 1989). The reason for the discrepancy is not known but may again be related to the hydration status, because i.c.v. administered saline induced natriuresis, which may contract extracellular volume. Furthermore, evidence suggests that ANP_{95-126} may be the substance responsible for the increase in sodium excretion during the central hypertonic stimulation. When a selective 2% increase in the sodium concentration of the carotid blood was produced by infusions of hypertonic saline, a 34-fold increase in renal sodium excretion concomitant with a 4-fold increase in the rate of urinary excretion of ANP_{95-126} was noted (Emmeluth et al., 1992).

Recently, Charles et al. (1991a) studied the ANP secretory response to acute volume expansion (15 ml/kg dextran during 30 min) in five sheep given nonimmune serum and ANP antiserum by i.c.v. injections. After antiserum treatment, the plasma IR-ANP response to dextran loading was significantly reduced, whereas loading caused similar decreases in hematocrit and increases in central venous and mean arterial pressure (Charles et al., 1991a). These results support the hypothesis that the brain ANP system is important in the ANP response to volume loading. The mechanism whereby i.c.v. ANP antiserum inhibited volume expansion-induced ANP secretion is not clear, but ANP antibody injected into the lateral cerebral ventricles may interfere with the function of ANP neurosecretory fibers and their projections to key endocrine and autonomic cell bodies (Charles et al., 1991a).

Clearly, further work is required to define the role of neural activity and brain areas possibly involved in the modulation of ANP secretion in response to different stimuli. Based mainly on in vitro studies and some experimental data in vivo, long-term activation of the parasympathetic system, if any, would be expected to inhibit basal and stimulated ANP secretion, whereas the secretory response may be facilitated by an increase in the sympathetic tone. The experimental findings that specific brain areas, especially the posterior pituitary are possibly involved in ANP release, remain to be tested critically.

G. Other Secretagogues

1. Hypoxia and hypercapnia. Hypoxia has been shown to stimulate ANP secretion both in vivo and in vitro. In vivo, acute and chronic hypoxia stimulate IR-ANP release in rats (McKenzie et al., 1986; Stockmann et al., 1988; Jin et al., 1989; Winter et al., 1989; Raffestin et al., 1990; Stewart et al., 1991), rabbits (Baertschi et al.,

1988), pigs (Adnot et al., 1988; Baertschi et al., 1990), lambs (Baertschi and Teague, 1989), and dogs (Vachiery et al., 1990). In humans, hypoxia (10% O_2, 4.5% CO_2) and high altitude (du Souich et al., 1987; Bärtsch et al., 1988; Lawrence et al., 1990; Lawrence and Shenker, 1991) also elevate plasma IR-ANP concentration. Alveolar hypoxia (pO_2 = 37 mm Hg) was as potent as 15% blood volume expansion in increasing circulating IR-ANP concentrations in conscious lambs, and importantly, prior volume expansion potentiated the hypoxia-induced elevation of plasma IR-ANP concentration (Baertschi and Teague, 1989). In vivo release may be mediated by pulmonary vasoconstriction, as suggested by the development of right ventricular hypertrophy (McKenzie et al., 1986; Stockmann et al., 1988; Winter et al., 1989; Raffestin et al., 1990; Chen et al., 1991) and by the increase of pulmonary arterial pressure and pulmonary vascular resistance (Baertschi and Teague, 1989; Baertschi et al., 1990; Raffestin et al., 1990; Vachiery et al., 1990). In support of these experimental findings, plasma IR-ANP levels were elevated in patients with chronic pulmonary artery hypertension in proportion to the level of pulmonary arterial pressure and pulmonary vascular resistance (Adnot et al., 1989). However, hypoxia has also been reported to increase plasma IR-ANP levels without correlation with hypoxia-induced pulmonary changes (Clozel et al. 1989; Vachiery et al. 1990). Increased pulmonary artery stretch may cause ANP secretion via a neural reflex loop, because lesions of the cervical vagosympathetic trunks decreased the ANP response to hypoxia by 45 to 58% (Baertschi et al., 1990). Right and left atrial pressures, heart rate, and blood volume also increase in response to acute or chronic hypoxia and may thus also contribute to elevated plasma IR-ANP levels (Baertschi and Teague, 1989; Baertschi et al., 1990; Raffestin et al., 1990). This is supported by the observation that hypoxia (paO_2 ≥45 mm Hg) in conscious dogs did not affect plasma IR-ANP concentration when blood pressure and left and right atrial pressure remained unchanged (Clozel et al., 1989). The decrease in right atrial IR-ANP levels in response to chronic hypoxia (see section III) is also consistent with the suggestion that atrial stretch, as a consequence of pulmonary vasoconstriction, may be involved in the hypoxia-induced ANP release. Furthermore, metabolic factors, blood volume, and increased secretion of catecholamines and vasopressin may also contribute to the hypoxia-induced ANP release.

Hypoxia may have a direct effect on ANP release in vitro in the isolated perfused rat and rabbit heart (Baertschi et al., 1986; Lew and Baertschi, 1988). Hypoxia-induced IR-ANP release from the heart is dependent on the severity of hypoxia and is both reversible and repeatable. Both α- and β-adrenergic mechanisms may mediate 35 to 40% of the IR-ANP response to hypoxia in isolated hearts, because phentolamine, propranolol, or catecholamine depletion by 6-hydroxydopamine each reduced peak hypoxia-induced ANP release, whereas atropine had no effect (Lew and Baertschi, 1988). Myocyte stretch increases myocardial oxygen consumption, and tissue hypoxia may represent the underlying mechanisms for ANP release.

In summary, hypoxia is a potent stimulus for ANP release in vitro and in vivo. Atrial stretch, increased heart rate, α- and β-adrenergic mechanisms, neural reflexes, and metabolic factors may mediate the effect of hypoxia on ANP secretion (Lew and Baertschi, 1988; Baertschi and Teague, 1989; Baertschi et al., 1990). Thus, in addition to its role in volume and pressure homeostasis, ANP may mediate adaptive responses to acute and chronic hypoxia. The enhanced release of ANP in response to elevated pulmonary artery pressure induced by hypoxia may be viewed as an appropriate physiological response, because ANP by decreasing pulmonary vasoconstriction may limit the increased pressure load developed on the right ventricle and thus alleviate right heart failure and pulmonary edema.

2. Myocardial ischemia and metabolic changes. Myocardial ischemia is a potent stimulus for ANP release in vivo. Increased plasma IR-ANP levels have been found in patients with ischemic heart disease and myocardial infarction (Lang et al., 1986; for reviews, see Weidmann et al., 1989; Sagnella and MacGregor, 1990) as well as in response to transient left ventricular ischemia induced by percutaneous transluminal coronary angioplasty (Gasser et al., 1989; Ikäheimo et al., 1989). Myocardial ischemia-stimulated ANP release is commonly thought to be mediated by increased atrial distension caused by ventricular failure. Elevated levels of IR-ANP in ischemia have been shown to correlate positively with ventricular dysfunction and elevated atrial pressure (Lang et al., 1986; Ikäheimo et al., 1989). However, experimental studies in vitro suggest that myocardial ischemia and coronary blood flow may directly affect ANP release. Naruse et al. (1987) showed in the isolated perfused rat heart that IR-ANP release can be stimulated by increasing perfusion pressure and coronary flow rate, implicating a role for flow in the release of ANP. Perfusion with 50 mM KCl produced immediate cardiac arrest and decreased IR-ANP release to an undetectable level with a significant decrease in coronary flow (Naruse et al., 1987). However, ANP release has been suggested to be independent of moderate changes in flow rate (6 to 15 ml/min); although an increase in the perfusate flow rate reduced the IR-ANP concentration, there was no difference in the rate of IR-ANP release (Klair et al., 1989). In conscious dogs, the increase in ANP secretion after 500 ml of volume expansion was due to the increase in coronary blood flow because the concentration of IR-ANP in blood leaving the atria increased only slightly (Hintze et al., 1990).

The correlations between myocardial redox and energy

states and ANP release have been studied in the perfused rat heart during global or low-flow ischemia for varying periods (Uusimaa et al., 1992c). Reduction in coronary flow produced a rapid decrease in IR-ANP release, ischemia/reperfusion stimulated IR-ANP release into the perfusate, and the increase in IR-ANP release during the postischemic period showed a positive correlation with atrial lactate to pyruvate ratio and a negative correlation with the atrial phosphorylation potential. In agreement with the enhanced release of ANP after global ischemia, low-flow ischemia also increased IR-ANP release (Uusimaa et al., 1992c). The negative correlation with the energy state and the positive correlation with the reduction of cytosolic NAD in response to ischemia/reperfusion and the hypoxia-induced ANP release described above suggest that ANP release from the heart may be linked to changes in myocardial energy metabolism. The intracellular mechanism of ANP release induced by ischemia is not yet clear but may be related to changes in several intracellular signaling pathways caused by ischemia/reperfusion.

3. Osmolality. Arjamaa and Vuolteenaho (1985) first described enhanced IR-ANP release from atrial cells in vitro due to increased osmolality. Using isolated, spontaneously beating rat atria in an organ bath, they found that both hyperosmotic NaCl and choline chloride solutions increased IR-ANP release. Hyperosmotic NaCl had no effect on the processing of ANP, because ANP_{99-126} was released into the incubation medium from isolated perfused paced atria (Arjamaa, 1989). In support of these studies, the IR-ANP secretion from atrial tissue fragments (Naruse et al., 1986) or enzymatically dispersed perfused adult rat atrial cells (Gibbs, 1987b) was stimulated by increases in extracellular osmolality, regardless of the solute (NaCl, KCl, or glucose); this secretory effect did not require extracellular Ca^{2+} (Gibbs, 1987b). In a recent study, extracellular hyperosmolality induced by NaCl and KCl (390 to 490 mOsm) enhanced IR-ANP release from superfused atrial slices (Zongazo et al., 1991). In contrast, others found no change in IR-ANP release when the osmolality was elevated in a rat heart-lung (Dietz, 1987) or isolated rat atria preparation (Agnoletti et al., 1990c). Furthermore, high concentrations of NaCl moderately increased IR-ANP release in the heart-lung preparation, but this was attributed to increases in atrial pressure (Onwochei and Rapp, 1988).

The physiological role of hyperosmolality in the regulation of ANP release in vivo is controversial, mainly because increased osmolality and hypervolemia cannot be separated from each other under physiological conditions; an increase in sodium tends to isoosmotically expand intravascular blood volume. However, a significant increase in plasma IR-ANP levels has been found in rats after infusion of hypertonic NaCl (Eskay et al., 1986; Kato et al., 1986a; Morris and Alexander, 1988). Hypertonic sodium load in conscious newborn calves (Amadieu-Farmakis et al., 1988) and in the bovine fetus (Amadieu-Farmakis et al., 1989; Miner et al., 1990) also increased plasma IR-ANP concentrations. Furthermore, significant diurnal variation of plasma IR-ANP levels, serum osmolality, and hematocrit occur in the rat (Kanervo et al., 1991). However, increases in plasma IR-ANP concentration (76%) in conscious dogs during hypertonic saline infusion producing an 11% increase in plasma osmolality appear to be induced by increases in right atrial pressure (Salazar et al., 1986a). Furthermore, an increase in plasma sodium did not increase circulating IR-ANP in conscious dogs when changes in left atrial and mean arterial pressures were transient and small (Nishida et al., 1988c) or remained unaltered (Emmeluth et al., 1990). In humans, Kamoi et al. (1988) also suggested that the secretion of ANP is indirectly modulated by osmotic pressure.

The increase in plasma osmolality may be the signal for ANP release in early vertebrates (for a recent review, see Evans, 1990). Various investigators have described IR-ANP histochemically in atrial and ventricular tissue of several fish species (Chapeau et al., 1985; Reinecke et al., 1985, 1987a,b; Westenfelder et al., 1988; Evans, 1990). Cultured fish atrial and ventricular myocytes secrete IR-ANP into the incubation medium, and the IR-ANP that is secreted may be structurally similar to the mammalian peptide (Baranowski and Westenfelder, 1989). IR-ANP is present in the plasma of species of all three major groups of fishes (Agnatha, Chondrichthyes, Osteichthyes) (Westenfelder et al., 1988; Evans, 1990). IR-ANP levels in fish living in higher saline environments (1% NaCl) were significantly higher than the circulating IR-ANP levels measured in freshwater fish (Westenfelder et al., 1988). Furthermore, circulating IR-ANP levels increased (Westenfelder et al., 1988) and decreased (Westenfelder et al., 1988; Evans, 1990) when fishes were acclimatized to high and low aquarium water salinity, respectively. A high correlation was found when serum sodium concentration was plotted against plasma IR-ANP levels (Westenfelder et al., 1988). Because hyponatremia produces volume loading and hypernatremia produces hypovolemia in teleosts (Evans, 1990), the changes in serum osmolality rather than intravascular volume appear to determine ANP release in these experiments.

In summary, there is some evidence that ANP-containing granules may respond directly to changes in systemic electrolytes such as sodium and chloride. However, because hyperosmotic challenge in conscious animals rapidly shifts fluid into the vascular space, the involvement of the atrial stretch response cannot be excluded. Yet, a recent study by Arjamaa et al. (1992) suggests that osmolality may contribute to the regulation of plasma IR-ANP concentration together with the right atrial pressure. During hypertonic saline infusion, when right atrial pressure was kept constant by lowering the

legs of the subject resting in the supine position, an increase in plasma IR-ANP concentration was noted in subjects with normal serum water conditions but not in dehydrated subjects (Arjamaa et al., 1992). Similarly, Burrell and Baylis (1990) observed an increase in plasma IR-ANP concentration after infusion of hypertonic saline when subjects were supine but not when they were seated. In support of these findings, water deprivation in rats, despite increasing serum osmolality, decreases plasma IR-ANP concentration (Januszewicz et al., 1986b). Thus, these data suggest that hyperosmolality does not stimulate ANP release when animals or subjects are dehydrated and intravascular volume is low.

4. Opiates. Horky et al. (1985) first reported that morphine in high doses in conscious rats produced a 25-fold increase in plasma IR-ANP concentration. Although later pharmacological experiments have established that morphine and other opiates produce their ANP-releasing action through an interaction with specific opiate receptors (opiate-induced ANP release could be reversed completely by naloxone and naltrexone) (Gutkowska et al., 1986c; Vollmar et al., 1987; Crum and Brown, 1988; Szecowka et al., 1990), the exact mechanism by which opiates induce ANP release into the circulation is not known. The type of opiate receptor mediating the ANP response is also controversial because both μ- and κ-receptor agonists have been shown to affect ANP levels. The μ-agonist fentanyl as well as morphine caused 8- to 10-fold increases in the plasma IR-ANP concentration in conscious rats (Gutkowska et al., 1986c; Vollmar et al., 1987), and a selective κ-receptor agonist U 50488-H was ineffective (Vollmar et al., 1987). In contrast, in urethane-anesthetized rats, i.v. administration of dynorphin A_{1-10} amide, a κ-agonist, produced a 12-fold increase in plasma IR-ANP concentration which was only blocked by high doses of naloxone (Tang et al., 1987; Xie et al., 1988), whereas in lower doses in conscious animals dynorphin, as well as leu-enk and β-endorphin, were inactive (Crum and Brown, 1988).

There is some evidence that opiates may exert their action on plasma IR-ANP concentration via a central mechanism, because 10-fold lower doses of morphine need to be administered i.c.v. than systemically to obtain the same increase in the plasma levels of IR-ANP (Crum and Brown, 1988; Chen et al., 1989), peripherally administered μ-receptor antagonist did not affect fentanyl-induced ANP release (Vollmar et al., 1987), and central but not peripheral administration of β-endorphin increased circulating IR-ANP levels (Szecowka et al., 1990). Pesonen et al. (1990) examined the mechanism by which fentanyl, a μ-receptor agonist, stimulates the release of ANP into the circulation by measuring plasma IR-ANP levels and hemodynamics (mean arterial pressure, heart rate, right atrial pressure) in conscious rats after i.v. administration of fentanyl. In conscious rats, i.v. infusion of fentanyl at doses of 3 and 10 μg/kg produced an immediate decrease in heart rate with little or no alteration in blood pressure. The effect of fentanyl on the right atrial pressure was dependent on the dose of fentanyl used; the lowest dose of fentanyl had no effect, and 3 μg/kg of fentanyl decreased right atrial pressure, whereas an increase was always noted when the highest dose of fentanyl was infused; the elevation in atrial pressure in response to fentanyl was comparable to that reported after volume expansion by saline under identical experimental conditions (Ruskoaho et al., 1989b,c). Thus, the opiate-induced increase in right atrial pressure probably explains the effect of infusion of high doses of opiates on plasma IR-ANP levels, because the marked increase in plasma IR-ANP levels in response to fentanyl infusion was associated with a prominent increase in right atrial pressure. However, Hoffman et al. (1989) reported that the administration of fentanyl (25 μg/kg bolus, followed by 50 or 200 μg/kg infusion for 30 min) did not influence basal or volume load-stimulated plasma IR-ANP concentrations in anesthetized rats, although it produced an immediate increase in blood pressure. The reason for this discrepancy is not known but might relate to the time of blood sampling; the samples for ANP determination were taken 30 min after infusion of fentanyl in the study of Hoffman et al., and peak IR-ANP concentrations are attained 5 to 10 min after fentanyl injections and return to control levels within 30 to 40 min (Vollmar et al., 1987).

Although an increase in atrial pressure would seem to explain the effect of high doses of fentanyl on ANP release, other mechanisms also appear to be involved in opioid-stimulated ANP release. Infusion of fentanyl at a dose of 3 μg/kg increased plasma IR-ANP levels (30 to 35%) but simultaneously decreased right atrial pressure significantly (Pesonen et al., 1990). The fact that central infusions of morphine and other opiates increase plasma levels of IR-ANP more than i.v. infusions (Crum and Brown, 1988; Chen et al., 1989) suggests that the effect of opiates on plasma IR-ANP concentration may in part be mediated through actions on the central nervous system. Furthermore, the finding that a smaller amount of IR-ANP was released in the vasopressin-deficient conscious Brattleboro rats (homozygous for hereditary genetic hypothalamic diabetes insipidus) than in the normal conscious LE rats for a given increase in right atrial pressure (Pesonen et al., 1990) suggests that the ANP secretory effects of opiates may be due in part to changes in vasopressin release. Finally, the attenuated response to fentanyl in the DI rats as well as the stimulatory effect of lower doses of fentanyl with a decrease in right atrial pressure further supports the hypothesis that other factors than atrial stretch modulate the release of ANP into the circulation.

In vitro studies have revealed a direct stimulatory effect of opiates on IR-ANP release also, suggesting that endogenous opioids may be involved in the regulation of ANP secretion. Dynorphin, a κ-agonist, at a relatively

high concentration, but not met-enkephaline (Lachance et al., 1986; Ferrari and Agnoletti, 1989), directly stimulated the release of IR-ANP from isolated rat atria (Stasch et al., 1989a). Furthermore, dynorphin stimulated IR-ANP release dose and time dependently from 2-day-old cultured atrial cardiocytes (Yamada et al., 1991). The dynorphin induced IR-ANP secretion was partially antagonized by MR2266, a selective κ-opioid antagonist, and U-62066E, a selective κ-opioid receptor agonist, stimulated IR-ANP secretion (Yamada et al., 1991), suggesting that dynorphin stimulates ANP secretion via activation of a specific κ-opioid receptor. In summary, opiates produce a marked stimulatory effect on the secretion of ANP in conscious rats. At higher opiate doses, the increased ANP release after opiate infusion appears to be mediated by hemodynamic changes, but other factors including direct effects on atrial myocytes may also contribute to the opiate-induced ANP release. However, the role of ANP in the renal and cardiovascular effects of opiates as well as the direct role of opiates in regulating ANP release in experimental animals and humans remains to be determined.

5. Other peptides. Recent studies in which immunohistochemistry was used have demonstrated a peptidergic innervation of the myocardium and the coronary circulation. Nerve fibers immunoreactive to neuropeptide Y, vasoactive intestinal peptide, somatostatin, substance P, and CGRP have been identified in the heart (Wharton et al., 1988b), and some of them have been suggested to be involved in the control of ANP secretion. CGRP, which may serve as a neurotransmitter in atrial tissue, stimulates the release of IR-ANP from isolated rat atria (Yamamoto et al., 1988a; Schiebinger and Santora, 1989). Calcitonin itself failed to stimulate IR-ANP release (Schiebinger and Santora, 1989), suggesting that ANP secretion may be modulated by nerve fibers containing CGRP. Furthermore, capsaicin treatment, which abolished CGRP and substance P immunoreactive nerve fibers from the heart, inhibited IR-ANP release induced in vivo by volume loading and in isolated perfused rat hearts by balloon inflation of the right atrium (Rankin and Scott, 1990). Infusion of CGRP increased plasma IR-ANP concentration in healthy normotensive volunteers (Gennari et al., 1991). Bolus injection of thyrotropin-releasing hormone also caused a gradual increase in plasma IR-ANP concentration in humans (Sergev et al., 1990).

It has been shown that neuropeptide Y produces a significant increase in plasma IR-ANP levels when injected i.v. into rats, whereas polypeptide Y decreased plasma IR-ANP and attenuated the volume expansion-induced increase in plasma IR-ANP levels (Baranowska et al., 1987c). Substance P also increased circulating IR-ANP levels in conscious rats after i.v. injection, whereas vasoactive intestinal peptide and neurotensin decreased plasma IR-ANP levels and blood pressure (Baranowska et al., 1987c). In addition, platelet-activating factor, a lipid mediator distributed and released by a variety of cell types, stimulated IR-ANP release from the isolated perfused rat heart and following i.v. injection in conscious normal rats (Rayner et al., 1991). The platelet-activating factor receptor antagonist BN52021 attenuated this stimulated release, suggesting a role for platelet-activating factor in ANP secretion. Finally, administration of corticortropin-releasing factor induced a short-term increase in IR-ANP release in the isolated perfused rat heart preparation (Grunt et al., 1992).

6. Other stimuli affecting atrial natriuretic peptide release. A number of other stimuli have been reported to influence plasma IR-ANP levels in vivo. These include snack (Homcy et al., 1985), licorice (Forslund et al., 1989), potassium supplementation (Barden et al., 1991), heat exposure (Finnish sauna) (Leppäluoto et al., 1991), cold (Hassi et al., 1991), alcohol (Colantio et al., 1991; Leppäluoto et al., 1992), snoring (Partinen et al., 1991), immobilization stress (Horky et al., 1985), acute footshock stress (Blizard and Morris, 1987), shaker stress (Yamamoto et al., 1989), carotid chemoreceptor stimulation (Al-Obaidi et al., 1991), hibernation (Zatzman and Thornhill, 1989), and diving (Baeyens et al., 1989; Vesely et al., 1991). Most likely the changes in right and left atrial pressures as well as other factors summarized earlier may explain altered plasma IR-ANP levels during these stimuli. Diurnal variation of plasma IR-ANP concentration (Leppäluoto et al., 1990) and higher plasma IR-ANP levels in old than young subjects (Ohashi et al., 1987a,b; Clark et al., 1991) and in aged rats (Imada et al., 1985; Kato et al., 1987; Korytkowski and Ladenson, 1991; Kao et al., 1992) presumably are also related to the hemodynamic and hormonal factors mentioned above.

Acute hypercalcemia is a potent stimulus for ANP release in vivo. Yamamoto et al. (1988b) showed that acute infusion of calcium chloride into anesthetized dogs caused an increase in circulating IR-ANP levels. The observation was later confirmed (Fujimura et al., 1989; Zawada et al., 1990). Mean plasma IR-ANP concentrations were significantly higher in the SHR and WKY rat after $CaCl_2$ infusion in the absence of significant changes in mean arterial blood pressure in calcium-infused animals (Fujimura et al., 1989). On the other hand, calcitriol, $1,25(OH)_2D_3$, the active metabolite of vitamin D_3, given intraperitoneally 3 h before experiment, was reported to reduce IR-ANP release from isolated rat atria (Wong et al., 1991a). The increase in plasma IR-ANP concentration was less pronounced in parathyroidectomized rats compared with intact rats (Geiger et al., 1990), suggesting that serum levels of parathyroid hormone may play a role in the ANP secretion. α-Difluoromethyl-ornithine, which inhibits ornithine decarboxylase activity and polyamine synthesis, decreased both basal and AVP-stimulated levels of IR-ANP in rats (Tipnis and Boor, 1992). The administration of putrescine restored the levels of

IR-ANP, suggesting that the polyamine pathway affects ANP secretion. Finally, adenosine$_1$ receptor agonist 2-chloro-N^6-cyclopentanyl adenosine (Polidori et al., 1990) and several drugs (ACE inhibitors, β-adrenoceptor antagonists, diuretics, calcium channel antagonists, nonsteroidal anti-inflammatory drugs, contrast agents) have been reported to influence plasma IR-ANP levels in vivo. Obviously, the hemodynamic and hormonal changes and other factors explain altered plasma IR-ANP levels after administration of these agents.

H. Regulation of Ventricular Release of Atrial Natriuretic Peptide

Early in vitro studies showed that neonatal ventricular cardiocytes in culture secrete IR-ANP into the incubation medium (Bloch et al., 1986; Cantin et al., 1987). As has been discussed, pressure and volume overload resulting in ventricular hypertrophy are characterized by augmentation of ventricular synthesis and storage of ANP. The recent studies have also shown that the cardiac ventricular source contributes significantly to the circulating ANP levels. The proportion of ANP secreted from atria and ventricles has been evaluated by studying IR-ANP secretion into perfusate from the hearts of SHR and cardiomyopathic hamsters by the Langendorff method both before and after atrialectomy (Ruskoaho et al., 1989a; Thibault et al., 1989c). Results of these studies clearly showed that basal IR-ANP release was increased from hearts with myocardial hypertrophy. Furthermore, atrialectomy had a lesser relative effect on perfusate concentration of IR-ANP in hypertrophic hearts in SHR; at least 28% of the total amount of IR-ANP released originated from ventricles in the SHR and only 8% in the age-matched 1-year-old WKY rat without hypertrophy (Ruskoaho et al., 1989a). In the hamster with severe CHF, as much as 74% of IR-ANP released into the perfusion fluid is produced in the ventricles (Thibault et al., 1989c). Furthermore, there is a significant correlation between ventricular weight and ANP release in vitro (Kinnunen et al., 1990), showing that the amount of the peptide released depends on the degree of ventricular hypertrophy.

Ventricular release of ANP has also been seen in vivo. Physical exercise in SHR and WKY rats was used as an experimental model to stimulate release of ANP from the heart, and ventricular levels of IR-ANP were measured at rest and after exercise (Ruskoaho et al., 1989a). A 30-min swimming exercise in SHR and WKY rats caused marked increases in cardiac work load and plasma IR-ANP concentrations with significant decreases in left (24 to 34%) and right (24 to 39%) ventricular IR-ANP concentrations. The depletion of ventricular IR-ANP was greatest in the SHR in the endocardial layer of the left ventricular wall (30%) where the intramyocardial pressure is known to be highest (Ruskoaho et al., 1989a). Although the amount of ANP released during swimming was greater in the SHR, the percentage of increase in plasma IR-ANP concentration was similar in the WKY rats (240%) when compared to that in the SHR (233%), suggesting that the hypertrophic process in SHR does not alter the ability of ventricular cells to secrete ANP in response to physiological stimuli in this experimental model of cardiac hypertrophy. In humans, increased basal ventricular ANP release has been seen in patients with DCM (Yasue et al., 1989).

Thus, the results convincingly show a link among myocardial hypertrophy, ventricular synthesis/storage, and release of the ANP from the heart. However, the physiological stimulus for ventricular ANP release is not clear. As in the atria, the principal stimulus controlling the acute release of ANP from the ventricles may be wall stretch. In support of this, an association between ventricular IR-ANP content and increase in plasma IR-ANP levels in response to acute volume expansion in the conscious hypertensive rat strain was observed (Ruskoaho and Leppäluoto, 1988a). Furthermore, the stimulated release of ANP in vivo occurred mainly from the endocardial layer of the left ventricle (Ruskoaho et al., 1989a) where the intramyocardial pressure is known to be highest. The finding that, at any atrial pressure or in response to phenylephrine, hearts of hypertensive Dahl-S rats released more IR-ANP than hearts of normotensive rats (Onwochei et al., 1987; Onwochei and Rapp, 1988) could also mean that increased afterload increased ANP release from the ventricle. The findings of a 74% reduction in the right ventricular IR-ANP concentration after 2 h of hypoxia resulting in pulmonary vasoconstriction (Winter et al., 1989) and a decrease in the left ventricular IR-ANP concentration in response to acute mitral regurgitation within 4 h in dogs (Satoh et al., 1990) also suggest that the ventricular cells are capable of responding to pressure overload. The large decrease in right ventricular immunoreactivity after 2 h was not accompanied by an increase in plasma ANP levels (Winter et al., 1989), probably reflecting the 1000-fold lower concentration of IR-ANP in the nonhypertrophied ventricle compared with the atria. Furthermore, the effect of cardiac overload caused by performing suprarenal aortic coarctation resulted in ANP release from the ventricular cardiocytes, as indicated by a decrease in number and migration of the granules toward the cell membranes (Gu et al., 1989). Several other studies, summarized in section III, of the effects of increased pressure or volume overload both in humans and in animals have demonstrated enhanced left or right ventricular ANP biosynthesis. Taken together, these observations suggest that stretch could be a common hemodynamic stimulus for the reexpression and release of ANP within the ventricular myocardium.

To test the hypothesis that stretch releases ANP from ventricular myocytes, Kinnunen et al. (1992) determined the ability of normal and hypertrophic ventricular tissue

in the isolated perfused heart preparation to secrete IR-ANP in response to varying degrees of stretch produced by the dilation of a balloon in the left ventricle. The animals used were old SHR and WKY rats. Using a modification of the isolated perfused heart preparation, which permitted gradual mechanical distension of the left ventricle, Kinnunen et al. (1992) found that stretching of the ventricular myocytes in arrested, isolated, atrialectomized perfused rat hearts caused a pressure-dependent, rapid increase in IR-ANP release into the perfusate, confirming that ANP is also a ventricular hormone stimulated by increased cardiac filling pressure. The high-performance liquid chromatography analysis of the perfusates showed that the ANP-like immunoreactive material released corresponded to the processed, active, low molecular weight peptide found in plasma, suggesting that the release of the peptide by ventricular distension is a physiological mechanism and is not the result of nonspecific tissue destruction. The observation that only one major single peptide was released from hypertrophied ventricles of SHR contrasts with the results obtained from experiments on normal and cardiomyopathic hamsters, in which a relatively large amount of intact proANP was released by the ventricles (Thibault et al., 1989c). Thus, ANP processing in ventricles may be species dependent. In addition to stretching, ischemia (Uusimaa et al., 1992b) and ET (Uusimaa et al., 1992a) have been reported to release IR-ANP from rat ventricular cells in vitro.

How stretching of ventricular myocytes or other stimuli increase ANP secretion remains to be established. Originally, Bloch et al. (1986) suggested that neonatal ventricular cells utilize a constitutive pathway for ANP release because they lack the secretory granules characteristic of atrial cardiocytes which store the peptide before secretion. Furthermore, the ratio of the ANP level to the ANP mRNA level (ANP/ANP mRNA) in the ventricle is smaller than that in the atrium of normal and hypertrophic hearts. These observations support the possibility that ANP is secreted more rapidly after synthesis from the ventricular cardiomyocytes. However, the presence of ANP granules in the hypertrophied ventricular tissue (Ding et al., 1987; Edwards et al., 1988a; Kinnunen et al., 1991) suggests that a secretagogue would enhance ANP release from hypertrophied ventricular cells in a similar manner to that occurring in atria. In fact, phorbol esters that stimulate PKC activity increase IR-ANP release from severely hypertrophied SHR ventricles but not from normal rat myocardium (Kinnunen et al., 1991). These results demonstrated the ability of mature ventricular tissue to secrete ANP in response to a secretagogue and suggested a possible role for PKC in the regulation of basal ANP secretion from ventricular cells, as reported previously in atria (Ruskoaho et al., 1985). However, the time course for the stretch-stimulated IR-ANP release differed markedly from that observed in atria (Ruskoaho et al., 1986a), because the maximal stimulation of ventricular IR-ANP release occurred during the first 2 min of stretching, and this was followed by a gradual decrease in IR-ANP release during the 10-min stretch period and reached control values by the end of it (Kinnunen et al., 1992). In the atria, as in the ventricles, the IR-ANP release increases rapidly in response to stretching but remains constant or continues to increase during stretching (Ruskoaho et al., 1986a). This difference probably reflects the more limited ANP storage capacity of the ventricular tissue and differences in mechanisms of ANP secretion between atria and ventricles.

In conclusion, in ventricular hypertrophy, ANP is synthesized, stored, and released from ventricular and atrial cardiocytes (fig. 6), reflecting the higher demand for ANP secretion when cardiac workload increases. As occurs in the atria, increases in cardiac filling pressure that increase ventricular myocardial wall stretch have now been shown to cause release of active, low molecular weight ANP-like peptide already identified in plasma. The kinetics of the ANP secretory response to stretch suggest that the ventricles make a greater contribution to the circulating ANP level at the onset of cardiac pressure or volume overload. However, if the overload persists, the atrial contribution to ANP release becomes

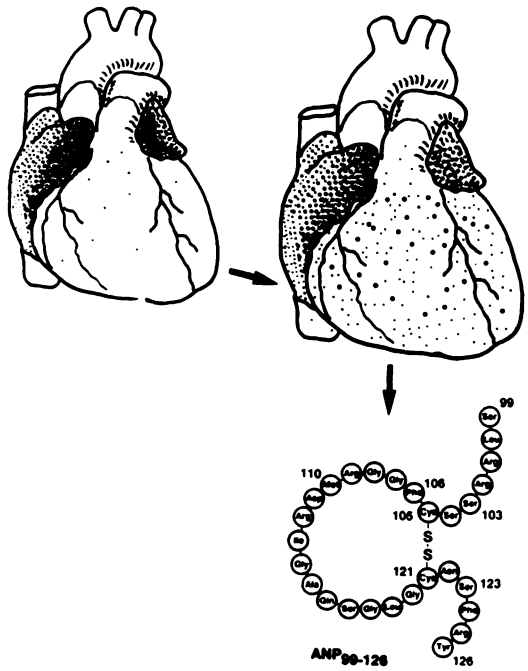

FIG. 6. Participation of cardiac ventricles in the regulation of ANP secretion from the heart. Cardiac ANP gene expression, storage, and release in pressure and volume overload: increased ANP gene expression in atrial and, especially, ventricular myocytes; reduced atrial and markedly increased ventricular IR-ANP concentration; atrial and ventricular myocytes release ANP_{99-126} into the circulation; stretching of atrial and ventricular myocytes increases ANP secretion; the amount of ANP released from ventricles is related to the degree of ventricular hypertrophy; and PKC activation by phorbol esters stimulates ANP release from atrial and hypertrophic ventricular cells.

more important. Thus, ANP is also a ventricular hormone.

I. Cellular Signals Regulating Atrial Natriuretic Peptide Secretion

As we have seen, atrial wall stretch is a major factor in the regulation of the release of ANP; however, the precise cellular mechanisms linking mechanical distension as well as other extracellular signals to hormonal release have not been fully established. One of the reasons for this is that in the study of ANP secretion experimental approaches aimed at understanding exocytosis are complicated by the fact that the exocytotic process is regulated by several interacting second-messenger systems (Ruskoaho et al., 1987, 1991; Glembotski et al., 1991). This means that manipulations of cells that modify secretion may not necessarily do so directly at the level of the exocytotic machinery. However, considerable progress has recently been made in understanding the organization of the ANP secretory pathway.

Cellular export of proteins can occur via two secretory pathways, called regulated and constitutive pathways (Lingappa, 1989). Proteins secreted by the regulated pathway are stored in secretory granules and are released in response to an appropriate stimulus, whereas peptides secreted by the constitutive pathway are released rapidly as soon as they are produced. As summarized earlier, ANP is stored in atrial myocytes as well as in hypertrophied ventricular cells in granules, which suggests that its secretion is regulated. The temperature dependence of ANP secretion (Bilder et al., 1986; Agnoletti et al., 1990c; Page et al., 1990) implies the involvement of energy-requiring reactions or processes such as protein kinases C and A and Ca^{2+}/calmodulin kinases in hormone secretion.

By analogy to other secretory systems in which release of hormones and peptides are regulated (Rasmussen, 1986), secretion of ANP may also be a Ca^{2+}-dependent process. However, unlike other endocrine cells, cardiocytes are also contractile, and the release and reuptake of intracellular Ca^{2+} are necessary for their normal systolic and diastolic function (Reiter, 1988; Morgan, 1991). Excitation-contraction coupling is initiated when depolarization permits Ca^{2+} to enter the myoplasm through the voltage-dependent calcium channels in the sarcolemma. This calcium releases a much larger quantity of activator Ca^{2+} from intracellular stores in the sarcoplasmic reticulum, initiating the cardiac contraction. Relaxation occurs when Ca^{2+} dissociates from the contractile apparatus and is resequestered by the calcium pump of the sarcoplasmic reticulum. The synchronous function of these steps allows the heart to maintain a 10,000-fold concentration gradient for Ca^{2+} across the cell membrane. Other important regulatory systems in the mammalian heart include the second messengers cAMP, IP_3, and DAG, which modulate the intracellular [Ca^{2+}] and, thus, the contractility through the activation of protein kinases. It is obvious that extracellular signals such as stretch and neurohumoral agonists or different pharmacological probes used in studies of ANP secretion may also directly or indirectly alter the handling of Ca^{2+} and, thus, myocardial function. This fact must even be considered when ANP secretion is studied using primary neonatal or adult cells in culture.

1. Role of phosphoinositide hydrolysis and protein kinase C. An important mechanism for regulating cellular physiology involves activation of phosphoinositide hydrolysis with subsequent production of IP_3 and DAG. IP_3 stimulates the release of sequestered Ca^{2+} into the cytoplasmic compartment (Berridge, 1987), whereas DAG causes the association of PKC with the plasma membrane and the conversion of PKC to an active, Ca^{2+}-sensitive protein kinase (Nishizuka, 1986). Sustained cellular responses mediated by the phosphoinositol system are thought to involve a sustained increase in the DAG content of the plasma membrane and in the activity of the membrane-associated PKC. The combined functions of these second messengers, IP_3 and DAG, act synergistically in regulating the function of many endocrine organs (Nishizuka, 1986; Berridge, 1987).

PKC has been characterized and purified from the heart (Kuo et al., 1984), where it is present in both membrane and cytosolic fractions in atrial and ventricular myocytes (Kuo et al., 1984; Yuan and Sen, 1986). The properties of PKC in cardiac myocytes are similar to those reported in other tissues (Kuo et al., 1984), and its activity appears to be higher in the atria than in the ventricles (Wrenn et al., 1988). Intracellular activation of PKC in vitro and in vivo can be achieved by tumor-promoting phorbol esters such as TPA and phorbol 12,13-didecanoate, which are structurally similar to DAG. The activation of PKC is probably brought about by the binding of phorbol esters to the same sites normally occupied by DAG (Nishizuka, 1986). PKC has been shown to be activated by phorbol esters in both isolated perfused hearts (Yuan et al., 1987) and isolated cultured myocytes (Henrich and Simpson, 1988; Irons et al., 1992).

Several lines of evidence support the concept that PKC activation may promote ANP secretion from the heart. Originally, the phorbol esters TPA and phorbol 12,13-didecanoate were shown to stimulate IR-ANP secretion in the perfused, spontaneously beating rat heart, and the relative potencies for stimulating ANP secretion by phorbol esters were the same as described earlier for activating PKC in vitro (Ruskoaho et al., 1985). TPA combined with the Ca^{2+} ionophore A23187 (Ruskoaho et al., 1985), Ca^{2+} channel agonist Bay K8644 (Ruskoaho et al., 1986b), adenylate cyclase activator forskolin (Ruskoaho et al., 1986b), or an α_1-agonist, methoxamine (Ruskoaho and Leppäluoto, 1988b), produced a synergistic effect. Additionally, a phorbol ester analog, 4α-phorbol 12,13-

didecanoate, which is incapable of binding to and activating PKC, was also inactive as an ANP secretagogue (Ruskoaho et al., 1985). These observations led to the concept that ANP secretion may be regulated by the PKC system.

Furthermore, at concentrations similar to those active in intact cells, TPA also causes IR-ANP release from cultured neonatal atrial (Fukuda et al., 1988; Matsubara et al., 1988a; Shields and Glembotski, 1989; Uusimaa et al., 1990; Irons et al., 1992) or adult (Iida and Page, 1988, 1989) myocytes. In adult cells, TPA stimulated IR-ANP secretion without requiring Ca^{2+} influx or ryanodine-inhibitable release of Ca^{2+} from the sarcoplasmic reticulum, although the absolute magnitude of TPA-stimulated IR-ANP concentrations was greater for the contracting cells (Iida and Page, 1989). In neonatal atrial myocytes, phorbol esters had a synergistic effect with ionomycin (Matsubara et al., 1988a) or Bay K8644 (Irons et al., 1992), giving further support for calcium-activated PKC in ANP secretion. Incubating cultures for >1 h with TPA resulted in a blunted secretory response, consistent with the down-regulation of PKC by prolonged treatment with phorbol ester (Shields and Glembotski, 1989; Irons et al., 1992).

Also, data obtained from numerous studies indicate that activators of phosphoinositide hydrolysis stimulate ANP secretion in a similar way to phorbol ester activation. In cardiocytes, α_1-adrenergic (Brown et al., 1985), ANG II (Allen et al. 1988), ET (Shubeita et al., 1990; Irons et al., 1992), vasopressin (Jard, 1985), and muscarinic-cholinergic receptors (Brown et al., 1985) are coupled to phosphoinositide hydrolysis and when stimulated probably cause the DAG-mediated stimulation of PKC (Nishizuka, 1986). As described before, results of most studies indicate that α_1-adrenergic agonists are able to stimulate release of ANP in different experimental models, including isolated perfused hearts, isolated atria, and cultured cells. Interestingly, the temporal pattern of IR-ANP secretion from the isolated perfused rat heart (Ruskoaho and Leppäluoto, 1988b) and atria (Schiebinger et al., 1987) to α_1-agonists resembles that observed for PKC activation. Catecholamines have been shown to account for approximately half of the hypoxia-induced IR-ANP release in the isolated perfused rat heart (Lew and Baertschi, 1988) and may mediate frequency-stimulated IR-ANP release under certain conditions (Bilder et al., 1989b). A calmodulin antagonist, W-7, and a PKC inhibitor, H-7, have been reported to inhibit phenylephrine-stimulated IR-ANP release (Ishida et al., 1988), further supporting the role of phosphoinositide hydrolysis and PKC in regulating ANP secretion. Yet, TPA and polymyxin B and neomycin did not significantly affect pacing-induced IR-ANP release from isolated perfused rat heart (Doubell, 1989b).

Less consistent results have been observed with other activators of receptors coupled to phosphoinositide hydrolysis in cardiac cells, although in many experimental models cholinergic agonists, ANG II and vasopressin, have also been reported to increase ANP secretion. Furthermore, as discussed before, these hormones and neurotransmitters have been reported to have no effect on or even to inhibit (cholinergic agonists) ANP secretion in different experimental models. Finally, although in most experimental models α-adrenergic receptor stimulation increases ANP release, this has been difficult to reproduce in some studies. The conflicting data existing concerning ANP secretion by agonists stimulating phosphoinositide hydrolysis may be due to methodological differences. In support of this, Sonnenberg et al. (1989) showed that, when isolated atria are studied, the method of preparation of atrial tissue is critical for the secretory effect of ANG. The finding that the inositol phosphate accumulation induced by ANG II and vasopressin has been shown to be less than that induced by α_1-adrenergic agonists (Berridge, 1987) might be one explanation for the different results. The failure of all hormones and neurotransmitters that activate phosphoinositide hydrolysis in heart cells to stimulate ANP secretion may also be partially explained by the different effects of receptor agonists on other intracellular second messengers, probably modulating the PKC activity and IP_3 response (Nishizuka, 1986; Berridge, 1987). For example, acetylcholine, acting via muscarinic receptors, stimulates phosphoinositide hydrolysis, but it depresses contractility by inhibiting adenylate cyclase and by activating K^+ channels (Birnbaumer et al., 1990). Thus, ANP secretion appears to be stimulated by many compounds activating the phosphoinositide hydrolysis, suggesting that this pathway may be involved in the secretory mechanism of ANP.

2. Cytosolic Ca^{2+} homeostasis and atrial natriuretic peptide secretion. Changes in cytosolic free [Ca^{2+}] concentration constitute an important element of signal transduction in various cells. These changes either reflect alterations in Ca^{2+} fluxes or result from mobilization of intracellular Ca^{2+} (Rasmussen, 1986). In the myocardial cell, a number of factors favor a physiological role for [Ca^{2+}] in modulating ANP secretion. First, ANP secretion is regulated by factors that increase either atrial stretch or the rate of atrial contractions, as mentioned before. Each of these factors has been linked to changes in intracellular [Ca^{2+}] in muscle cells (Tsien, 1983; Morgan, 1991). Under normal physiological conditions in cardiocytes, neurotransmitters and humoral agents may promote mobilization of intracellular Ca^{2+} stores by stimulating phosphoinositide hydrolysis and formation of IP_3, a potent stimulus for Ca^{2+} mobilization (Reiter, 1988). In addition, Ca^{2+} influx and the release of Ca^{2+} from intracellular Ca^{2+} stores are two processes that play important roles in the mechanisms leading to hormone secretion by endocrine cells (Shambrook, 1990). In cells with a regulated secretory pathway, secretory granules

fuse with the cell membrane in response to a cytoplasmic messenger, such as increased [Ca^{2+}]. Interestingly, the secretory granules in atrial myocytes contain large amounts of calcium (Somlyo et al., 1988), further supporting the possibility of an association between intracellular [Ca^{2+}] and ANP secretion.

Studies in which different experimental models of ANP release were used have shown that certain experimental manipulations or drugs that affect the concentration of intracellular Ca^{2+} influence ANP release. The Ca^{2+} ionophore A23187, which introduces free Ca^{2+} into the cell, increased IR-ANP secretion in the isolated spontaneously beating rat hearts (Ruskoaho et al., 1985). Furthermore, the combination of A23187 and phorbol ester stimulated IR-ANP secretion more than the calculated additive value for each agent. A23187 or ionomycin have also been reported to increase IR-ANP release from neonatal cultured atrial myocytes (Bloch et al., 1988; Matsubara et al., 1988a; Greenwald et al., 1989; LaPointe et al., 1990).

Results of other studies in which various probes were used support the view that an increase in cytosolic [Ca^{2+}] may affect ANP secretion. The dihydropyridine derivative Bay K8644, which increases calcium current by direct action on voltage-dependent Ca^{2+}-channels, has been reported to increase IR-ANP secretion in spontaneously beating perfused hearts (Ruskoaho et al. 1986b; Saito et al., 1986) or electrically paced atria (Schiebinger, 1989; Schiebinger and Santora, 1989) and in isolated myocytes (Matsubara et al., 1988a; Irons et al., 1992). Under conditions in which a large majority of cells contracted spontaneously, the concentration of IR-ANP accumulated increased as the external [Ca^{2+}] (range 0.2 to 1.8 mM) was increased (Iida and Page, 1989). In isolated myocytes, nifedipine and verapamil, drugs known to decrease intracellular [Ca^{2+}] in heart cells, inhibited IR-ANP secretion (Iida and Page, 1989; LaPointe et al., 1990), and the effect of Bay K8644 on IR-ANP secretion was blocked by nifedipine (Saito et al., 1986; Matsubara et al., 1988a). Ryanodine, which abolished spontaneous contraction, suppressed IR-ANP accumulation into the incubation medium (Iida and Page, 1989). Depolarization of atrial cells by KCl produced a dose-dependent release of IR-ANP in isolated myocytes (Greenwald et al., 1989; Sei and Glembotski, 1990). Furthermore, nifedipine and verapamil completely inhibited KCl-stimulated IR-ANP secretion and partially inhibited phenylephrine-induced IR-ANP secretion from cultured neonatal myocytes (Shields and Glembotski, 1989). The change in IR-ANP response to phenylephrine was completely inhibited by Ca^{2+} depletion in the perfused rat heart (Toki et al., 1990). Similarly, in the isolated superfused rat left atria preparation, decreasing the superfusate [Ca^{2+}] and nitrendipine inhibited phenylephrine-stimulated IR-ANP secretion, whereas ryanodine had no effect (Schiebinger et al., 1992). A low concentration of Ca^{2+} (1.2 mM), nifedipine, W-7, and ryanodine were reported to decrease basal IR-ANP release from isolated perfused rat heart (Katoh et al., 1990). Ouabain, which in rat myocardial cells increases intracellular [Ca^{2+}], stimulates IR-ANP secretion from rat atrial cardiocytes (Bloch et al., 1988) and superfused left atria (Hu et al., 1988b; Schiebinger and Santora, 1989). Acute elevation of perfusate [Ca^{2+}] caused increased IR-ANP release from rat atria (Wong et al., 1991b). The increase in IR-ANP secretion from the perfused rat heart induced by rapid cardiac pacing was abolished by nifedipine, nisoldipine, and verapamil, and when nifedipine and nisoldipine were infused alone, both decreased basal IR-ANP release into the perfusate (Doubell, 1989a,b). Results of these numerous experiments suggest that calcium ions may play an important role in stimulus-secretion coupling of atrial cells, as has been shown in many other endocrine organs.

In contrast, when either isolated cells that do not contract or arrested atria and hearts (inhibited by chemical compounds) are examined, different results have been obtained. In noncontracting primary cultures of atrial myocytes from adult rats, neither an influx of extracellular Ca^{2+} nor a release of Ca^{2+} from ryanodine-sensitive intracellular stores in the sarcoplasmic reticulum is required for the substantial and constant rate of IR-ANP secretion (Iida and Page, 1989). When spontaneously beating rat atria were used, ethyleneglycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid and Ca^{2+}-free media induced a significant increase in the rate of basal IR-ANP release (de Bold and de Bold, 1989; Kuroski-de Bold and de Bold, 1991). Introduction of 49 mM KCl resulted in a significant decrease in IR-ANP release in nominally Ca^{2+}-free and 0.625 nM Ca^{2+} medium, but not at higher [Ca^{2+}] (Kuroski-de Bold and de Bold, 1991), suggesting that changes in Ca^{2+} levels do not affect IR-ANP release in media containing Ca^{2+} in the range of physiological concentrations. Under these experimental conditions, ryanodine, caffeine, and Ba^{2+} had no effect, and La^{3+} decreased and Sr^{2+} increased IR-ANP release from isolated rat atria (Kuroski-de Bold and de Bold, 1991). Moreover, forskolin, cAMP analogs, or a phosphodiesterase inhibitor isobutylmethylxanthine, which all elevate the intracellular [Ca^{2+}], have been reported to reduce IR-ANP secretion in nonbeating (contraction inhibited by tetrodotoxin) adult atrial cardiocytes (Iida and Page, 1988). In the isolated perfused rat heart, 8 mM KCl did not affect IR-ANP release (Baertschi et al., 1986), whereas 50 mM KCl produced cardiac arrest and a significant decrease in IR-ANP release (Naruse et al., 1987). Sonnenberg et al. (1984) reported that KCl decreased ANP release from atrial tissue fragments analyzed by bioassay.

In recent years, new methods that allow direct measurement of intracellular [Ca^{2+}] have emerged, including the development of the fluorescent Ca^{2+} indicators quin-2 and fura-2. Uusimaa et al. (1990) evaluated the role of

calcium as a second messenger of ANP secretion by using fura-2 to measure intracellular [Ca^{2+}] following various stimuli. For simultaneous observations of cytosolic [Ca^{2+}] and ANP secretion, myocardial cells cultured on microcarriers were packed in a chromatography column. IR-ANP secretion was found to remain unchanged in spite of considerable transient increases in [Ca^{2+}] induced by KCl or Bay K8644, suggesting that Ca^{2+} is not the main mediator of ANP release under these experimental conditions (Uusimaa et al., 1990). Instead, PKC activation by TPA stimulated ANP secretion from the cultured neonatal rat atrial myocytes, which confirmed the role of activation of PKC in ANP release.

In summary, the rate of contraction or tension, processes modulated by variations in cytoplasmic [Ca^{2+}] concentration (Tsien, 1983), may explain the discrepant effects of experimental manipulations affecting intracellular [Ca^{2+}] on ANP secretion. It appears that, when spontaneously beating or paced isolated atria or hearts are studied, enhanced calcium influx is able to modestly augment ANP secretion. If isolated cultured myocytes, which in general beat slower or are arrested, are used, no effect or even a decrease in ANP secretion has been seen when procedures known to increase intracellular [Ca^{2+}] are performed. Furthermore, the observation that short-term neonatal atrial cell cultures secrete the hormone inefficiently with no stimulation of secretion by KCl (Sei and Glembotski, 1990) may in part be responsible for the discrepant results. Thus, although primary cardiac cultures serve as a useful model system for studies of ANP release by allowing the secretory response in the absence of neural, hormonal, or hemodynamic stimuli to be studied, both the contractile behavior of cells and the culture time requires quantification when the results of experimental manipulations are interpreted. In support of this, abolishing contraction with 10 μM tetrodotoxin significantly reduced IR-ANP accumulated in the incubation medium from adult atrial myocytes (Iida and Page, 1989). Clearly, experiments with paced cultures with direct estimations of the cytoplasmic Ca^{2+} concentration and the ANP secretory rates will be required to assess these discrepancies.

Furthermore, the findings of an increased IR-ANP secretion observed in several studies after omitting Ca^{2+} with or without ethyleneglycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid treatment may explain the alterations of cellular cytoskeletal proteins, probably leading to enhanced secretory granule migration to the cell membrane or enhanced exocytosis. The Ca^{2+} depletion in these studies does not appear to have been cytotoxic, because IR-ANP secretion returned to baseline when Ca^{2+} was returned to the buffer. The transient increase in IR-ANP secretion upon the reintroduction of Ca^{2+} (de Bold and de Bold, 1989) may be due to a transient increase in cytosolic [Ca^{2+}]. Although, the results of the studies conducted in the absence of extracellular Ca^{2+} obviously do not reflect the normal physiological situation of contracting and relaxing atrial cells, they may yet be useful in understanding ANP secretion in certain special circumstances, including those observed in vivo when passive mechanical stretch of myocytes is low (i.e., hypotension, hemorrhage) or in the absence of contraction.

3. Role of cyclic adenosine 3′,5′-monophosphate and guanosine 3′,5′-monophosphate in atrial natriuretic peptide release. The cellular content of cAMP depends on a series of enzymatic steps, initiated by receptor stimulation and ending in cAMP degradation. An increase in [cAMP], by activating a cAMP-dependent protein kinase (protein kinase A), increases the force of contraction in cardiac cells by phosphorylating voltage-dependent calcium channel proteins and converting them from latent to voltage-responsive channels, thereby increasing Ca^{2+} influx into the cell (Reiter, 1988; Morgan, 1991). cAMP also facilitates Ca^{2+} uptake into the sarcoplasmic reticulum and Ca^{2+} extrusion through the sarcolemmal calcium pump, accelerating the relaxation of heart muscle (Reiter, 1988; Morgan, 1991). Thus, changes in levels of intracellular Ca^{2+} and in the contractile force may be achieved by interventions that act on cellular cAMP levels. For example, the formation of cAMP by activation of adenylate cyclase plays a decisive role in the inotropic effect of catecholamines (Reiter, 1988).

Several compounds are available that directly increase the levels of cAMP in the cell, including forskolin, an activator of adenylate cyclase, as well as membrane-permeable analogs of cAMP, such as dibuturyl-cAMP. When forskolin was tested for its ability to affect ANP secretion from the perfused rat heart, both the basal and TPA-stimulated IR-ANP secretion were increased (Ruskoaho et al., 1986b). Pretreatment with dibuturyl-cAMP also enhanced the phorbol ester-stimulated IR-ANP release (Ruskoaho et al., 1986b), and continuous superfusion with dibuturyl-cAMP produced a biphasic increase in IR-ANP secretion from paced left atria, an increase similar to that induced by isoprenaline (Schiebinger, 1988, 1989; Schiebinger and Santora, 1989). Thus, cAMP, previously shown to modulate the secretion of many endocrine cells, may be involved in controlling ANP secretion from atrial cardiocytes.

Neurohumoral agents or pharmacological drugs that increase the cAMP content of cardiac cells have also been shown to stimulate ANP secretion. In heart cells, β-adrenergic receptors on cardiocytes are coupled to adenylate cyclase, and isoprenaline, a β-adrenoceptor agonist, increased both basal and TPA-stimulated IR-ANP secretion from the perfused rat heart (Ruskoaho and Leppäluoto, 1988b). Similar results with β-agonists have been observed in other model systems of ANP release, including isolated atria (Schiebinger et al., 1987; Wong et al., 1988b; Ferrari and Agnoletti, 1989; Schiebinger and Santora, 1989) and dispersed atrial cells

(Gibbs, 1987b). However, the pattern and mechanism of the secretory response by each appears to be different; the initial secretory response to β-receptor stimulation develops more rapidly relative to the α-adrenergic response (Schiebinger et al., 1987; Ruskoaho and Leppäluoto, 1988b) and resembles that of forskolin and cAMP derivatives (Schiebinger, 1988; Schiebinger and Santora, 1989). The mechanism of the CGRP-stimulated ANP secretion appears to depend on the activation of adenylate cyclase and production of cAMP (Schiebinger and Santora, 1989), and conversely, methacholine-induced inhibition of ANP secretion stimulated by isoprenaline (Schiebinger, 1988) is related to a decrease in cAMP levels.

However, some studies have shown that agonists increasing cellular cAMP levels or cAMP derivatives have no effect or may even decrease ANP secretion. When cultures of neonatal cardiocytes were studied, forskolin, cAMP analogs, and isobutylmethylxanthine, all agents known to increase [cAMP], inhibited basal (Shields and Glembotski, 1989; Yamada et al., 1991) and phorbol ester-stimulated IR-ANP secretion (Shields and Glembotski, 1989). Accordingly, in isolated adult atrial cells, isoprenaline, forskolin, isobutylmethylxanthine, and dibutyryl-cAMP decreased IR-ANP release into the incubation medium (Iida and Page, 1988; 1989). When the cells were treated with ethyleneglycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid and ryanodine, the forskolin-induced inhibition was prevented (Iida and Page, 1988). Furthermore, forskolin and dibuturyl-cAMP had no effect on the release of IR-ANP from isolated rat atria (Inoue et al., 1988a). Isobutylmethylxanthine, which in combination with $PGF_{2\alpha}$ in the atrial cell culture increased cAMP levels, reduced $PGF_{2\alpha}$-stimulated IR-ANP release (Gardner and Schulz et al., 1990).

The physical state of the cells, atrial contraction rate and force against which the cells contract, probably explains this discrepancy. Cultured cells or nonbeating cells are not subject to the equal tension found in the contracting heart and generally beat at a relatively slow rate compared to that found in vivo. cAMP modulates intracellular [Ca^{2+}] through the activation of the protein kinases. Phosphorylation of the voltage-dependent Ca^{2+} channels increases influx of Ca^{2+} and thereby the amount of [Ca^{2+}] in the cell. As a consequence, IR-ANP secretion increases from spontaneously beating or paced atria with or without an increase in the force of contraction. cAMP also affects the diastolic relaxation of the heart, and phosphorylation of phospholamban, the regulatory subunit of the calcium pump, leads to a decrease in intracellular [Ca^{2+}] during diastole. Thus, because intracellular [Ca^{2+}] appears to be an important regulator of ANP secretion, an inhibitory effect of cAMP on ANP secretion is observed when cAMP does not cause an increase of [Ca^{2+}]; this appears to occur in cell culture or in nonbeating cardiac myocytes. In support of this, for example, isoproterenol failed to stimulate IR-ANP secretion by nonbeating left atria, and, in fact, IR-ANP secretion decreased (Schiebinger, 1989). These data suggest that β-agonists and compounds that increase [cAMP] may have both stimulatory and inhibitory effects on ANP secretion in vitro, depending on the experimental conditions used. This model would also explain the finding that isoproterenol also decreases IR-ANP secretion in vivo under some experimental conditions.

Information concerning the physiological function of cGMP as well as the cellular mechanisms that increase this cyclic nucleotide has increased considerably in recent years, but little information exists about the role of cGMP in the regulation of ANP secretion. The cGMP analog, 8-bromo-cGMP, or sodium nitroprusside, which induces cGMP formation in the myocardium, had no effect alone but delayed the phorbol ester-induced IR-ANP secretion from the perfused rat heart preparation (Ruskoaho et al., 1986b). Similarly, 8-bromo-cGMP slowed the rate of IR-ANP secretion in noncontracting cultured atrial myocytes (Iida and Page, 1988). Indirect evidence suggests that EDRF, which also increases cellular cGMP levels, inhibits IR-ANP secretion from isolated atria (Sanchez-Ferrer et al., 1990). Thus, an increase in cGMP may decrease ANP secretion from the heart.

4. Mechanisms of endothelin-induced atrial natriuretic peptide release. The potent stimulatory effect of ET has been very useful in clarification of the intracellular biochemical processes that may influence ANP secretion in response to certain extracellular stimuli. ET was originally proposed to produce vasoconstriction by acting as an agonist of voltage-dependent Ca^{2+} channels (Yanagisawa and Masaki, 1989). However, it is now clear that, after binding to cell surface receptors, ET activates several signal transduction pathways, including the activation of phospholipase C through interaction with a putative G protein. ET-1 modulates intracellular Ca^{2+} levels through both IP_3-dependent Ca^{2+} mobilization and opening of plasma membrane Ca^{2+} channels. In addition, ET causes other ionic and biochemical changes that contribute to cellular signaling. These changes include stimulation of phospholipase A_2, depolarization of membrane potential, activation of electroneutral Na^+-H^+ antiport, and inhibition of Na^+-K^+-ATPase (for reviews, see Masaki et al., 1991; Rubanyi and Botelho, 1991; Simonson and Dunn, 1991).

ET has been shown to be a potent ANP secretagogue in cultured myocytes (Fukuda et al., 1988), isolated rat atria (Hu et al., 1988a), and perfused rat hearts (Mäntymaa et al., 1990). In the perfused rat heart, ET is, on a molar basis, the most potent ANP secretagogue yet identified (Mäntymaa et al., 1990). First results from in vitro experiments suggested that ET may induce ANP release by acting on voltage-dependent Ca^{2+} channels. In cultured neonatal atrial myocytes (Fukuda et al., 1988)

and isolated rat atria (Hu et al. 1988a; Schiebinger and Gomez-Sanchez, 1990), low concentrations of the voltage-dependent Ca^{2+} channel antagonists (nicardipine, nitrendipine, verapamil) decreased the ET-induced IR-ANP release, whereas ryanodine had no effect (Schiebinger and Gomez-Sanchez, 1990). Furthermore, the ANP secretory response to ET by isolated atria was reduced by 65% with lowering of extracellular Ca^{2+} from 1.8 to 0.2 mM and to 51% in nonbeating atria compared with paced atria (Schiebinger and Gomez-Sanchez, 1990). Similarly, when free Ca^{2+} was reduced to 2 nM by adding 1 mM ethyleneglycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid to the low calcium medium in isolated atrial cultures, ET-induced IR-ANP secretion was partially inhibited (Sei and Glembotski, 1990). The time-course experiments revealed that the sustained phase of ET-induced ANP secretion appeared to be completely dependent on extracellular Ca^{2+}, whereas the initial phase of secretion displayed at best only a partial dependence on such Ca^{2+} influx. The finding that ET-induced ANP secretion is attenuated in nonbeating, nonstretched isolated atria (Hu et al., 1988a; Schiebinger and Gomez-Sanchez, 1990) further supports the notion that enhanced Ca^{2+} influx, through voltage-dependent calcium channels, is needed to support the maximal rate of ANP secretion by ET. Finally, when IR-ANP secretion and cytosolic [Ca^{2+}] was monitored simultaneously, ET-1 at concentrations of 1, 10, and 100 nM caused a concentration-dependent increase in [Ca^{2+}] and IR-ANP secretion in cultured neonatal atrial myocytes; the maximal increases in [Ca^{2+}] and IR-ANP secretion rate were 30 and 100% respectively (Uusimaa et al., 1992a). Yet, diltiazem, an antagonist of voltage-dependent Ca^{2+} channels, had no significant effect on ET-induced IR-ANP secretion (Uusimaa et al., 1992a).

The fact that ET-induced secretion was only partially inhibited, but not completely abolished by the above-mentioned experimental manipulations, indicates that other mechanisms in addition to extracellular Ca^{2+} influence ET-stimulated ANP secretion. Stimulation of neonatal rat myocardial cells with ET-1 induces a dose-dependent hydrolysis of phosphoinositides, resulting in the accumulation of [^{3}H]inositol phosphates including IP_3 (Shubeita et al., 1990). The hydrolysis of phosphatidylinositol in response to ET-1 was accompanied by a corresponding increase in DAG content. The accumulation of IP_3 peaked at 30 min, and a significant increase in DAG was seen 30 and 60 min after stimulation (Shubeita et al., 1990). Few investigators have studied the role of intracellular Ca^{2+} and IP_3 in the ET-stimulated secretion, and the results are conflicting. In isolated paced atria, ryanodine, an inhibitor of sarcoplasmic calcium release, failed to inhibit the ET-induced IR-ANP release (Schiebinger and Gomez-Sanchez, 1990). However, results from experiments in atrial cell cultures suggest that secretion is to some degree dependent on IP_3 and intracellular [Ca^{2+}]. The early phase of ET-stimulated IR-ANP secretion was inhibited when intracellular Ca^{2+} stores were depleted by ionomycin in medium containing 2.0 nM calcium and 1 μM nifedipine (Sei and Glembotski, 1990). Furthermore, pretreatment with LiCl (10 mM), which has been shown to interfere with cellular inositol pools, amplified the response to ET (Gardner et al., 1991). Thus, these studies suggest that atrial myocytes require both intra- and extracellular calcium to support maximal rates of stimulated secretion; the intracellular Ca^{2+} may be mainly used during the initial phase of agonist stimulated secretion, whereas extracellular Ca^{2+} is used during both the initial and sustained phases of ANP release. Furthermore, ET-mediated ANP secretion appears to depend on a calmodulin-dependent step, because a calmodulin antagonist, calmidazolium, inhibited both basal and ET-induced IR-ANP release into incubation media from isolated atrial myocytes (Gardner et al., 1991). Similarly, the Ca^{2+}/calmodulin kinase inhibitor KN-62 inhibited ET-stimulated IR-ANP secretion from neonatal rat atrial myocytes (Irons et al., 1992). Meclophenemate and pertussis toxin had no effect on the ET-stimulated release (Gardner et al., 1991), suggesting that PGs and pertussis toxin-sensitive G-proteins are not essential for ET to increase ANP release.

As discussed before, the activation of phosphoinositide by ET-1 is accompanied by an increase in DAG content, which directly activates PKC. ET has been shown to stimulate PKC in cultured vascular smooth muscle cells, and there is recent evidence that PKC may function as an intracellular mediator of ANP secretion of the peptides of the ET family. In the isolated perfused rat heart preparation (Mäntymaa et al., 1990; Pitkänen et al., 1991), the kinetics of the effects of ET-1 and sarafotoxin-b on IR-ANP release as well as on coronary vasculature and cardiac muscle resemble those of the phorbol ester TPA, which is known to mimic the action of DAG by directly activating PKC. Furthermore, ET augmented the phorbol ester-induced IR-ANP secretion (Fukuda et al., 1988; Mäntymaa et al., 1990), which is consistent with the observations that increases in cytosolic [Ca^{2+}] potentiates PKC activity (Nishizuka, 1986). This potentiating effect of ET on phorbol ester-induced ANP release resembled the action of the dihydropyridine Ca^{2+} channel agonist Bay K8644 (Ruskoaho et al., 1986b) and the calcium ionophore A23187 (Ruskoaho et al., 1985), supporting the hypothesis that calcium-activated PKC may play an important role in the mechanism of ANP secretion. Furthermore, in the perfused rat heart preparation, staurosporine, a potent inhibitor of PKC, at a concentration of 100 nM, which completely abolished the coronary vasoconstrictor effect of phorbol ester, attenuated the stimulatory effects of ET and sarafotoxin-b on IR-ANP release (Pitkänen et al., 1991). This inhibition was more apparent during the sustained phase (from 20

to 30 min) of ANP secretion stimulated by ET-1 and sarafotoxin. Similarly, staurosporine (10 nM) inhibited the sustained phase of ET-induced IR-ANP secretion from neonatal rat myocytes cultured on microcarriers (Uusimaa et al., 1992a). By monitoring the phosphorylation of a PKC substrate (p80) when exposed to ET, Irons et al. (1992) recently showed that ET activated PKC in primary atrial myocytes. Furthermore, down-regulation of PKC or exposure to a PKC inhibitor H-7 resulted in a 50% decrease in ET-stimulated IR-ANP secretion (Irons et al., 1992). Finally, complete inhibition of ET-mediated IR-ANP secretion from cultured neonatal rat atrial myocytes was obtained in the presence of the PKC inhibitor (H7) and Ca^{2+}/calmodulin kinase inhibitor (KN-62) (Irons et al., 1992). These results show that the DAG/PKC-signaling pathway may have an important role as an intracellular mediator of ANP secretion induced by peptides of the ET/sarafotoxin family.

In summary, the present data show that ET stimulates the hydrolysis of phosphoinositides to IP_3 and DAG, presumably activating PKC in cardiac myocytes to stimulate ANP secretion. Atrial cardiocytes appear to require both extracellular and intracellular Ca^{2+} to support maximal rates of ANP secretion in response to ET, and both intracellular Ca^{2+}-pools and IP_3 may be used during the early phase of secretion, whereas the extracellular source of Ca^{2+} and the activation of PKC may be important for the sustained phase of ANP secretion.

5. Mechanisms of stretch-induced atrial natriuretic peptide release. The atrial myocardium has a remarkably elastic structure and has been identified as a target of many mechanical stimuli via changes in pressure during contraction. Distension of the atrial myocardium also occurs as a result of a number of pathophysiological states, and this dilation results in the release of ANP. However, the cellular processes involved in linking mechanical distension to ANP release (mechanotransduction) have not been established. Observations by investigators using other cells exposed to an array of physical forces have led to the concept that the forces perceived by cells may dictate their shape, and the combined effects of external physical stimuli and internal forces responsible for maintaining cell shape may stimulate alterations in cellular biochemistry. Molecular structures that may be involved in mechanotransduction include stretch-activated and stretch-inactivated ion channels, Na^+/H^+ exchange, adenylate cyclase, and enzymes involved in phosphatidylinositol turnover (for review, see Watson, 1991), and some of them have also been suggested to contribute to stretch-mediated ANP release.

a. PHOSPHOINOSITIDE HYDROLYSIS. In the isolated perfused rat heart, stimulation of phosphatidylinositol turnover and formation of IP_3 was noted when right atria were dilated (von Harsdorf et al., 1988, 1989). This stimulation was detectable after 1 min with larger increases observed after 10 or 20 min, implying that phosphoinositol turnover could mediate the stretch-induced release of ANP. Ruskoaho et al. (1990) assessed the contribution of various second messengers to ANP secretion by using a modification of the perfused rat heart preparation that permits distension of the right atrium by pressures approximating those found in vivo to examine the mechanisms involved in atrial stretch-induced ANP release. A continuous infusion of vehicle or drugs was done for 35 min, and right atrial stretch was superimposed for 5 min after a 25-min perfusion by elevating the level of the pulmonary artery cannula tip. A mean increase in right atrial pressure of 2 mm Hg produced by elevation of the pulmonary artery cannula tip resulted in a 52% increase in the rate of IR-ANP release into the perfusate. When 10 to 24 nM TPA was added to the perfusion fluid, a dose-dependent augmentation of stretch-induced IR-ANP release was observed, i.e., for a given increase in the degree of stretch in the presence of phorbol ester, more ANP was released, whereas an inactive phorbol ester analog, 4α-phorbol 12,13-didecanoate, was also inactive as an ANP secretagogue (Ruskoaho et al., 1990). Thus, the activity of PKC appears to positively regulate stretch-induced ANP release. The observation that H-7, a PKC inhibitor, decreased the ANP secretory rate in isolated stretched atria (Page et al., 1990) is consistent with the role of PKC in control of ANP secretion.

Because TPA-mediated PKC activation appears to stimulate both basal and stretch-stimulated ANP release, hormones and neurotransmitters that activate phosphoinositide hydrolysis in heart cells would be expected to influence stretch-mediated ANP secretion. In fact, as discussed before, ET, α-adrenergic agonists, ANG II, vasopressin, and PGs have been shown to augment the atrial stretch-induced ANP release both in vivo and in vitro, although conflicting results have also been reported. H-7 decreased secretagogue-induced release but failed to inhibit ANP secretion induced by atrial stretch (Ishida et al., 1988). Additional work is thus required to clarify the effect of stretch on PKC activity in myocytes and to assess whether increased PKC activity may precede ANP secretion induced by stretch. Furthermore, nothing is known about the effect of stretch on the accumulation of DAG and its relationship to stretch-induced ANP secretion. Nevertheless, experiments comparing stretch, DAG levels, and PKC activation should provide very interesting information concerning the mechanisms of ANP secretion.

b. CELLULAR CALCIUM HOMEOSTASIS. Several studies have addressed the importance of cytosolic Ca^{2+} as a possible intracellular messenger mediating stretch-induced ANP secretion. Experiments have revealed both positive and negative modulation of stretch-induced ANP secretion by cellular Ca^{2+}. The evidence that Ca^{2+} may positively regulate stretch-induced ANP release includes the finding that the total IR-ANP released by

stretch has been shown to be partially suppressed in the calcium-depleted heart in the absence of spontaneous contractility (Ito et al., 1988). However, analysis of the molecular weight form revealed that the major ANP species released into Ca^{2+}-free medium was the high molecular weight prohormone (Toki et al., 1990). Cd^{2+} and Ni^{2+}, ions known to block Ca^{2+} currents through L- and T-type Ca^{2+} channels, respectively, significantly slowed the rate of IR-ANP secretion from isolated noncontracting rat atria (Page et al., 1990), suggesting that Ca^{2+}-selective channels are involved in control of stretch-dependent ANP secretion under some experimental conditions. A calmodulin-binding drug, trifluoperazine, inhibited the stretch-induced IR-ANP secretion in isolated rat atria (Page et al., 1990). Ryanodine pretreatment (10^{-6} M), which inhibits the release of Ca^{2+} from the sarcoplasmic reticulum, reduced stretch-induced IR-ANP release by 34%, in addition to inhibiting tension development and beating (Kuroski-de Bold and de Bold, 1991). In the isolated perfused heart, low concentrations of Ca^{2+}, nifedipine, W-7, and ryanodine significantly inhibited the basal and left atrial pressure-induced increase in IR-ANP secretion (Katoh et al., 1990). Finally, increasing extracellular Ca^{2+} concentration increased total (basal and stretch-activated) IR-ANP secretion from isolated rat atria in the absence of contractions (Page et al., 1991a), further suggesting that the increase in intracellular [Ca^{2+}] stimulates stretch-induced ANP secretion.

In contrast, results of several experiments have led to the conclusion that Ca^{2+} is a negative modulator of stretch-stimulated ANP release. The absence of Ca^{2+} from the perfusion medium increased total IR-ANP release and blocked the proteolytic cleavage of the prohormone (Ito et al., 1988). Ca^{2+} has been shown to negatively modulate secretion of IR-ANP from the isolated osmotically stretched neonatal atrial myocytes (Greenwald et al., 1988). Similarly, stretch-induced IR-ANP release in the isolated rat atrial preparation was independent of extracellular Ca^{2+} and took place even in the presence of ethyleneglycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (de Bold and de Bold, 1989; Kuroski-de Bold and de Bold, 1991). These experiments were carried out at 30°C on isolated spontaneously contracting rat atria suspended between two hooks, and abnormal cytology was observed in 15% of cardiocytes, with cell swelling being one of the most common cytological changes noted (de Bold and de Bold, 1989). The stretch-dependent IR-ANP secretion in isolated rat atria was present at a [Ca^{2+}] of 0.02 nM, and the atria could respond to stretch by an increase in secretory rate in spite of the absence of action potentials and atrial contractions produced by tetrodotoxin (Page et al., 1990). In a recent study, IR-ANP release from isolated rat atria induced by stretching was not inhibited by depolarization with KCl or a low concentration of external Ca^{2+} (Agnoletti et al., 1992). Furthermore, ryanodine-sensitive Ca^{2+} release was not essential for stretch-activated IR-ANP secretion in noncontracting rat atria (Page et al., 1990). In the perfused paced rat heart preparation, Bay K8644, a compound that increases the concentration of intracellular Ca^{2+}, also dose dependently inhibited the stretch-stimulated IR-ANP release, suggesting that Ca^{2+} may negatively modulate stretch-stimulated ANP release (Ruskoaho et al., 1990). Accordingly, a progressive decrease in the secretory rate of IR-ANP in isolated rat atria was noted; the effect was more marked at higher extracellular [Ca^{2+}] (Page et al., 1991a).

These observations indicate a difference in the stimulus-secretion coupling experienced by stretched and unstretched atria. When atrial myocytes beat spontaneously, drugs known to increase the concentration of intracellular Ca^{2+} moderately increase ANP release. But, when atria are stretched, increasing cytosolic [Ca^{2+}] appears to both stimulate and inhibit stretch-activated ANP secretion. These contrasting findings may be explained by time-dependent inhibition of [Ca^{2+}] on ANP release processes. Page et al. (1991a) examined the time course of stretch-dependent ANP secretion in the presence of different extracellular concentrations of Ca^{2+} in isolated atrial preparations and found an initial positive effect on ANP secretion. However, the second negative effect was more marked and became evident more rapidly as the extracellular [Ca^{2+}] was increased (Page et al., 1991a). This result supports that of Ruskoaho et al. (1990) who noted decreased secretory response to stretch after pretreatment with the Ca^{2+} channel agonist Bay K8644 for 25 min. Thus, [Ca^{2+}] appears to cause a transient positive modulation of stretch-induced ANP secretion, followed by inhibition. How this inhibition of secretion occurs remains to be clarified.

c. OTHER MECHANISMS. The role of other second messengers or ion transport processes in the regulation of stretch-dependent ANP secretion is still unclear. In the isolated perfused rat heart preparation, a 35-min infusion of forskolin, a compound that increases [cAMP] and then intracellular [Ca^{2+}], dose dependently inhibited stretch-stimulated IR-ANP release (Ruskoaho et al., 1990). Stretch-dependent IR-ANP secretion can also be inhibited by 8-chlorophenylthio-cAMP and caffeine in noncontracting rat atria (Page et al., 1990) and by isoprenaline pretreatment in contracting rat atria (Agnoletti et al., 1992), further suggesting that cytosolic [cAMP] may be a negative modulator of ANP secretion. By systematic deletion of external Na^+, K^+, Mg^{2+}, Cl^-, or HCO_3^-, and by selective ion transport inhibitors, transplasmalemmal fluxes of Na^+, Mg^{2+}, Cl^-, and HCO_3^- were not necessary for stretch augmentation of secretory rate in noncontracting atrial preparations at constant distending pressures of 5.1 mm Hg in the presence of 0.2 mM extracellular Ca^{2+}, 10 μM ryanodine, and either saxitoxin or tetrodotoxin (Page et al., 1990). Membrane

transporters selective for K^+ or relatively ion-nonselective channels could not be excluded in these studies because of the diversity of K^+ channels and methodological limitations (Page et al., 1990); yet, 4-aminopyridine- or tetraethylammonium-sensitive K^+ channels were not essential for stretch-dependent ANP release. Furthermore, inhibition of the Na^+-K^+-pump by ouabain had no effect on stretch-stimulated ANP release. Monensin, an Na^+-selective ionophore, has been reported to decrease the basal (Iida et al., 1988) and the atrial stretch-stimulated (de Bold et al., 1991) IR-ANP release, but monensin has also other effects, including effects on the translocation of secretion granules. Protein synthesis inhibition by cycloheximide for 44 min did not influence basal or stretch-induced IR-ANP secretion in noncontracting rat atria at a physiological external Ca^{2+} concentration (Page et al., 1991b), suggesting that the constitutive component does not contribute substantially to stretch-augmented ANP secretion. Brefeldin A, which disassembled Golgi cisternae, increased the stretch-augmented IR-ANP secretory rate in the noncontracting rat atria preparation and also prevented Ca^{2+}-dependent inactivation with time (Page et al., 1991b). Furthermore, cellular ATP depletion by carbonyl cyanide *m*-chlorophenylhydrazone rapidly and completely inhibited stretch-augmented IR-ANP secretion (Page et al., 1991b), consistent with the energy dependence of peptide secretion.

d. MYOCYTES OR OTHER CELLS AS SENSORS FOR STRETCH-INDUCED ATRIAL NATRIURETIC PEPTIDE RELEASE. When the mechanisms by which the atrial stretch is linked to the secretion of ANP are examined, it is also necessary to consider whether atrial wall stretch alone or factors liberated in response to distension are responsible for the activation of ANP secretion. The primary sensors for stretch-dependent ANP secretion in the atria may be cardiac myocytes; however, stretch sensors may also be localized in other cell types, including nerve cells, endothelial cells, endocardial cells, vascular smooth muscle cells, fibroblasts, or other specialized cells present in the atria. The nonmyocyte cells responsive to stretch could transmit the signal to the cardiac myocytes that results in augmented ANP secretion from myocytes. Theoretically, local release of acetylcholine or norepinephrine could influence ANP secretion, because they have been reported to affect ANP secretion under basal and stimulated conditions (see section IV.D). Yet, this seems unlikely, because addition of adrenergic or muscarinic cholinergic antagonists did not influence stretch-induced IR-ANP release (Schiebinger and Linden, 1986a; Agnoletti et al., 1992) and phosphoinositide turnover (von Harsdorf et al., 1989). Furthermore, the inability of tetrodotoxin and saxitoxin to prevent stretch-induced IR-ANP secretion from isolated rat atria (Page et al., 1990) also casts doubt on the presence of neuronal influences that affect stretch-augmented ANP secretion.

As discussed earlier, an exciting mechanism for the secretion of ANP in response to stretch is the release of stimulatory or inhibitory factors from the endothelium. ET, a vasoconstrictor peptide produced by endothelial cells, has been demonstrated to enhance atrial stretch-induced IR-ANP release from perfused rat hearts (Mäntymaa et al., 1990), cultured neonatal atrial myocytes (Gardner et al., 1991), and superfused rat left atria (Schiebinger and Greening, 1992). These in vitro observations suggest a possible regulatory role for the vascular endothelium in the regulation of ANP secretion in vivo (fig. 5) and are reminiscent of the modulation of smooth muscle function by EDRF and endothelium-derived contracting factors described in recent years (Katusic and Shepherd, 1991). The cellular mechanisms by which ET affects the atrial stretch-induced release of ANP remain to be elucidated. Stretch-activated channels have been described in endothelial cells, some of which are selective for Ca^{2+} (Watson, 1991). It has been suggested that sensitivity to stretch may be mediated through a cytoskeletal network that opens the channels when the cell membrane is stretched (Watson, 1991). Thus, ET released from endothelial cells in response to stretch could act together with Ca^{2+} to stimulate ANP release. The stimulation of calcium-activated PKC by ET might also explain the increased ANP secretion. Certainly, more data are needed concerning the mechanisms by which ET modulates cardiac ANP release.

In summary, these data suggest that the activation of phosphatidylinositol turnover and PKC, e.g., by ET and vasoconstrictors, may contribute to stretch-induced ANP release. The secretion of ANP in vitro in response to changes in intracellular [Ca^{2+}] and cAMP appears to depend on both atrial stretch and the rate of contraction, and calcium may have dual effects on cellular release of ANP by stretch. There is an initial transient stimulation followed by an inhibition as Ca^{2+} concentration increases.

6. Cellular signals regulating ventricular atrial natriuretic peptide secretion. Pressure and volume overload are characterized by augmentation of ventricular synthesis and storage of ANP, and as discussed earlier, studies in vitro and in vivo demonstrate that ANP is also released from ventricular tissue. The mechanism by which the ventricular cells secrete ANP is, however, not yet clear. Originally, Bloch et al. (1986) suggested that neonatal ventricular cells utilize a constitutive pathway for ANP release because they lack secretory granules characteristic of atrial cardiocytes, which store the peptide before secretion. They also reported that short-term exposure to the Na^+-K^+-adenosine triphosphatase inhibitor, ouabain, stimulates the secretion of IR-ANP from atrial, but not from ventricular, cardiocytes in culture (Bloch et al., 1988). Similarly, incubation of the neonatal ventricular cell cultures with phorbol ester did not alter the IR-ANP secretion rate (Shields and Glembotski, 1989; Uusimaa et al., 1990). Thus, neonatal ventricular

cardiocytes appear to utilize a constitutive secretory pathway, and a secretagogue would not be expected to enhance ANP release from this cell type. On the other hand, Greenwald et al. (1989) reported that KCl produced a dose-dependent release of IR-ANP from suspensions of neonatal rat ventricular cells and suggested that, like the atrial myocyte, the ventricular myocyte possesses the cellular mechanisms necessary to secrete ANP by a regulated pathway. The presence of ANP granules in hypertrophied ventricular tissue also suggests that a secretagogue would enhance ANP release at least from hypertrophied ventricular cells by the regulatory pathway analogous to that occurring in atria.

To determine in vitro the cellular mechanisms of ANP release from normal and hypertrophic ventricular cardiocytes, Kinnunen et al. (1991) studied the secretory effects of phorbol ester TPA in the SHR and WKY rats at the ages of 2 and 21 months by using the isolated, perfused heart preparation. In these experiments, TPA was added to the perfusion fluid for 30 min at a concentration of 46 nM after removal of atrial tissue. Interestingly, the considerable stimulation of ventricular IR-ANP secretion was only observed in hearts of old SHR with severe ventricular hypertrophy, high ANP gene expression and the presence of ANP containing granules (Kinnunen et al., 1991). In contrast, TPA had no effect on IR-ANP secretion from the ventricles of young animals. The fact that phorbol ester stimulates ANP release from the hypertrophied ventricles of hypertensive rats, but not from normal rat myocardium, shows the ability of mature ventricular tissue to secrete ANP in response to a secretagogue. Thus, with development of hypertrophy and the appearance of ANP-containing granules, ventricular cells appear to regain the ability to secrete ANP by the regulated pathway. The observation that the phorbol ester increases the release of ANP from the hypertrophied ventricle suggests that the PKC activity may be involved in the regulation of ANP secretion from ventricular cells, as occurs in atrial myocytes.

7. *Summary.* Pharmacological modulation of ANP secretory pathways in atrial and ventricular cardiocytes has provided new insights into the mechanisms regulating stretch- and secretagogue-induced ANP secretion. The experimental evidence appears to focus most consistently on the involvement of PKC and phosphoinositide hydrolysis in the stimulated release of ANP. Furthermore, under normal physiological conditions, PKC activation, Ca^{2+}, and cAMP have been shown to interact synergistically in the process of secretagogue-induced ANP release in spontaneously beating or paced atrial cells. Calcium, either through voltage- or receptor-mediated transplasmalemmal influx or released from the intracellular stores via IP_3 as a result of receptor-mediated activation of phospholipase C, may be sufficient to stimulate directly granular exocytosis. Calcium also may be capable of stimulating protein kinases that phosphorylate regulatory components of the atrial secretory apparatus. Depending on the secretagogue used, the kinetics of the response, as well as the second messengers involved in the regulation of the stimulated release of ANP, appear to be different. Furthermore, the same stimulus may activate several intracellular second messengers in a time-dependent manner to produce the final secretory response typical for each secretagogue. The activation of PKC probably plays a role during both the initial and the sustained phase of ANP secretion. PKC activation by phorbol esters also enhances stretch-stimulated ANP release, which agrees with observations that some potent activators of phosphoinositide hydrolysis enhance stretch-induced ANP release both in vivo and in vitro. The release of endothelial modulating factors may represent an intriguing new mechanism for the secretion of ANP in response to stretch. ET, one of these endothelial factors, has been suggested to enhance stretch-stimulated ANP release partly by activating PKC.

Further studies are needed to clarify the effect of stretch and secretagogues on the levels of cytoplasmic second messengers (Ca^{2+}, IP_3, DAG, cAMP, and cGMP) and ion channels and the activity of PKC to define whether changes in one or several of these intracellular systems precede ANP secretion. It is also important to clarify how heterologous stimuli such as myocyte stretch and hormones individually and in combination influence ANP secretion. Drugs that increase the intracellular concentration of calcium or cAMP have no effect or may even inhibit ANP secretion in vitro in noncontracting myocytes. It remains to be seen whether this situation resembles low atrial tension in vivo when a number of stimuli, including isoproterenol, fail to increase or even inhibit ANP secretion. Elucidation of the cellular mechanisms underlying stretch-mediated ANP secretion and the modulation of ANP secretion during changes in cardiac contractility should provide important insights into how ANP secretion is regulated. The interactions of cardiac myocytes and other cell types in the regulation of ANP secretion also need to be studied, and the mechanisms that modulate basal and stretch-induced ANP secretion need to be defined.

V. Metabolism of Circulating Atrial Natriuretic Peptide

The peptide structure of ANP predicts that its removal from the circulation is rapid and may occur via a number of metabolic clearance pathways. Preventing the elimination of ANP by inhibitors of these metabolic pathways may be a useful therapeutic tool for regulating endogenous ANP levels. However, the quantitative importance of these clearance pathways and how various inhibitors work has not yet been fully determined.

A. Pharmacokinetics of Atrial Natriuretic Peptide

After release into the circulation, ANP has a very short $t_{1/2}$, which is about 0.5 to 1 min in rats (Katsube et al., 1986; Luft et al., 1986a; Murthy et al., 1986a,b; Condra et al., 1988; Almeida et al., 1989; Krieter and Trapani, 1989; Chiu et al., 1991; Barclay et al., 1991), 0.5 min in mice (Gros et al., 1990a), 1 to 4 min in dogs (Verburg et al., 1986; Cernacek et al., 1988c; Woods, 1988; Nishida et al., 1990), 1 to 3 min in rabbits (King et al., 1989a; Marleau et al., 1989), 2 to 3 min in monkeys (Hoegler et al., 1989), and 2 to 5 min in humans (Gnädinger et al., 1986; Juppner et al., 1986; Nakao et al., 1986; Yandle et al., 1986b; Biollaz et al., 1987; Ohashi et al., 1987b). In the rat, radiolabeled ANP was cleared from the circulation with biphasic kinetics; the majority (90%) of ANP cleared with an initial $t_{1/2}$ of 7 to 33 s reflecting mixing and redistribution, and the remaining peptide was cleared with a $t_{1/2}$ of 2 to 5 min (Tang et al., 1984b; Murthy et al., 1986b; Chondra et al., 1988; Almeida et al., 1989; Widimsky et al., 1990b; Barclay et al. 1991; Pang et al., 1991; Widimsky et al., 1991). The slow phase is probably of minor importance because it accounts for <10% of the injected dose. In conscious rats, the total clearance was 90 to 150 ml/min/kg, representing about one-third to one-half of the normal cardiac output, and the V_{ds} (50 to 90 ml/kg) was slightly greater than the total blood volume of the rat (Almeida et al., 1989; Krieter and Trapani, 1989; Chiu et al., 1991; Widimsky et al., 1991). ANP's metabolic clearance rate (0.91 to 1.1 liters/min) is also very high in dogs (Verburg et al., 1986; Woods, 1988; Nishida et al., 1990), approaching the cardiac output of the dog. Plasma disappearance $t_{1/2}$ was 1.44 min during the rapid phase and 10.3 min during the slow phase of elimination, and the apparent V_{ds} during the steady state was 3.66 liters.

In humans, the plasma $t_{1/2}$ for the fast and slow components were 1 to 2 and 13 to 17 min, respectively (Gnädinger et al., 1986; Nakao et al., 1986; Ohashi et al., 1987b; Hensen et al., 1992). The initial phase was similar in both young and old subjects, whereas the second phase of ANP_{99-126} disappearance in the old group was prolonged to 34 min (Ohashi et al., 1987b). The prolongation of $t_{1/2}$ following termination of ANP infusion in the elderly was also observed by Clark et al. (1991). The metabolic clearance rate is about 13.6 to 25.4 ml/min/kg (2.5 to 9 liters/min), and the V_{ds} is 80 to 200 ml/kg in humans (Nakao et al., 1986; Yandle et al., 1986b; Ohashi et al., 1987b; Richards et al., 1989a; Moe et al., 1992; Tan et al., 1992). The total body clearance has been reported to be markedly reduced in elderly as compared with young subjects (Tan et al., 1992). Cardiac dysfunction may also affect ANP kinetics; the elimination $t_{1/2}$ was longer and the clearance rate slower in patients with CHF than in control subjects (Moe et al., 1992), although the calculated $t_{1/2}$ was not significantly different between patients with cardiac dysfunction and normal subjects in another study (Hensen et al., 1992). The dose of ANP used in infusion studies appears to affect the kinetics of ANP; in conscious rabbits, the kinetics were first order after bolus administration, whereas zero-order, dose-dependent kinetics were seen after prolonged infusion of ANP associated with a decrease in the systemic clearance and prolonged $t_{1/2}$ (Marleau et al., 1989). Posture alters ANP clearance, presumably by reducing the amount of ANP at clearance sites (Gillies et al., 1987). Early chromatographic analyses suggested that ANP may be partly bound to larger plasma proteins (Arendt et al., 1986; Stangl et al., 1986; Kato et al., 1988). In support of this, Wilson et al. (1991a,b) recently presented evidence that the large molecular form of IR-ANP ("big ANP"), present in the normal circulation of rabbits, rats, and humans, may be a carrier-bound form of ANP.

B. Elimination of Atrial Natriuretic Peptide by Different Tissues

ANP is eliminated from the circulation by almost every tissue (for a review, see Gerbes and Vollmar, 1990). After incubation of $ANP_{103-126}$ with tissue homogenates, Tang et al. (1984b) found the rank order of degradative potency to be kidney > liver > lung > plasma > heart. Weselcouch et al. (1985) reported that about 80% of the activity of crude atrial extract and synthetic $ANP_{103-125}$ was removed by isolated perfused rabbit kidney, and in vivo the dog kidney also removed about 80% of the activity of both of these substances. Little activity (14 to 26%) was lost when either crude atrial extract or $ANP_{103-126}$ was infused through the lungs (Weselcouch et al., 1985). The bolus infusion of synthetic $ANP_{103-126}$ in normal and nephrectomized rats suggested that the kidneys account for 59% of its elimination by degradation rather than excretion (Luft et al., 1986a). Others have also reported that nephrectomy may affect the elimination of ANP (Katsube et al., 1986; Barclay et al., 1991; Chiu et al., 1991).

The relative contributions of different organs to the extraction of ANP have been studied systematically in both experimental animals and humans by sampling their venous blood and comparing the IR-ANP concentrations to arterial levels. The lungs, kidneys, splanchic area/liver, and lower limbs each extract up to 50% or even more of the ANP in their arterial supply in humans (Crozier et al., 1986; Schutten et al., 1987; Vierhapper et al., 1988; Hollister et al., 1989; Bates et al., 1989; Vierhapper et al., 1990), dogs (Woods, 1988; Perrella et al., 1991b), and rats (Krieter and Trapani, 1989). Turrin and Gillis (1986) found a high uptake (67%) of injected ANP in a single pass through a perfused rabbit lung. In the conscious rat, the kidney (15 to 30%), intestine, and muscle/sex organs extracted a significant amount of $ANP_{103-126}$, whereas the liver and heart/lung only marginally affected metabolism (Krieter and Trapani, 1989). In dogs, the contribution of renal clearance to whole

body metabolic clearance rate was only 14%, and filtration of ANP across glomerular membranes accounted for 30% of the renal clearance (Woods, 1988). During ANP infusion, the splanchnic area and kidney both accounted for about 10% and leg vascular tissue 2.5% of the overall ANP clearance in human subjects (Vierhapper et al., 1988, 1990; Gasic et al., 1991).

When blood flow is taken into account, however, the lungs appear to be the most important site of elimination of ANP, demonstrating approximately 20 to 35% extraction of ANP (Bates et al., 1989; Hollister et al., 1989; Masuda et al., 1989; Obata et al., 1990; Northridge et al., 1992; Akaike et al., 1992); mean clearance rates of 634 to 840, 216, and 78 ml/min for lung, liver/splanchnic, and kidney circulations, respectively, were noted in human subjects (Hollister et al., 1989). When the disappearance of iodinated ANP_{99-126} was examined in rats, the highest uptake was found in the liver (16%), lung (14%), and kidney (12%); yet, bones, striated muscle, skin, and fat were calculated to account for 20 to 25% of total ANP uptake (Widimsky et al., 1990b). Thus, most organs are involved in the elimination of ANP. In descending order, lung, kidney, and liver/splanchnic area, followed by skeletal muscle and vasculature, are probably the principal organs for elimination in rats, dogs, and humans.

C. Pathways of Atrial Natriuretic Peptide Elimination

Receptor-mediated binding, uptake, and metabolism by target tissues, degradation by enzymes and other processes at plasma membranes, and excretion into nonplasma fluids such as urine may be involved in the ANP clearance from the circulation. The majority of ANP clearance probably involves a receptor-mediated endocytosis (Maack et al., 1987, 1988a,b; Nussenzveig et al., 1990a; Maack, 1992). ^{125}I-labeled ANP is initially bound to the cell membrane and then rapidly internalized and subsequently degraded (Hirata et al., 1985; Napier et al., 1986; Morel et al., 1987, 1988, 1989; Murthy et al., 1989; Nussenzveig et al., 1990b; Rathinavelu and Isom, 1991). Incubation of ANP with rat plasma caused only a slight loss in IR-ANP in the first 5 min (Murthy et al., 1986b), and metabolites of ANP appeared in the circulation after the majority (90%) of the hormone was cleared (Condra et al., 1988). Results of other studies also underscore the importance of ANP tissue uptake and binding for its rapid removal from the circulation (Maack et al., 1987; Almeida et al., 1989; Johnson et al., 1990; Nussenzveig et al., 1990b; Widimsky et al, 1990b). In vascular smooth muscle cells, the receptor-mediated clearance represented about 55%, whereas the extracellular proteolysis represented approximately 45% of the total clearance of ^{125}I-ANP_{1-28} (Johnson et al., 1990). Thus, receptor-mediated endocytosis with subsequent intralysosomal degradation and release of degradative products into the circulation plays a major role in the clearance of ANP and becomes a determinant of the mean clearance rate and V_{ds}. In addition to the clearance receptors, a number of studies have shown that ANP is eliminated from the circulation by enzymatic degradation and that the most important of the enzymes responsible for the degradation is NEP (endopeptidase EC 3.4.24.11, also called enkephalinase and atriopeptidase). Furthermore, enzymes of the renin-ANG system or kallikrein-kinin cascade and other metalloendopeptidases have also been implicated in ANP inactivation (fig. 7).

1. Receptor-mediated clearance of atrial natriuretic peptide. As with other peptide hormones, ANP exerts its effects by binding to specific membrane-bound receptors (de Lean et al., 1984; Hirata et al., 1984b; Napier et al., 1986; Leitman and Murad, 1986). Specific ANP-binding sites have been revealed by autoradiographic techniques in all ANP target tissues studied, including most notably adrenal, kidney, vasculature, and central nervous system (for reviews, see Brenner et al., 1990; Maack, 1992). Two main types of ANP-specific receptors have been identified in target tissues: guanylate cyclase-linked receptors (called ANP_A and ANP_B) and a receptor thought to have a clearance function (called ANP_C). The guanylyl cyclase family of proteins distribute to both particulate and soluble fractions of a cell, where they serve as receptors for various ligands, subsequently generating increased amounts of cGMP; two of them appear to be natriuretic peptide receptors, called ANP_A (GC-A) and ANP_B (GC-B) receptors (for review, see Chinkers and Garbers, 1991) (fig. 8). The ANP_A and ANP_B are approximately 1030-amino acid, monomeric 130-kDa proteins, and the deduced amino acid sequences suggest that both contain a single transmembrane domain that divides each protein

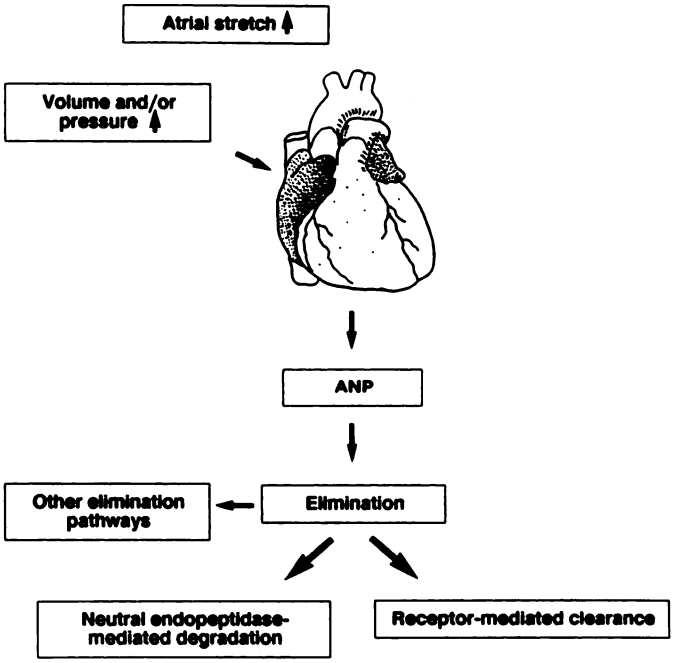

FIG. 7. Major pathways for elimination of ANP from the circulation.

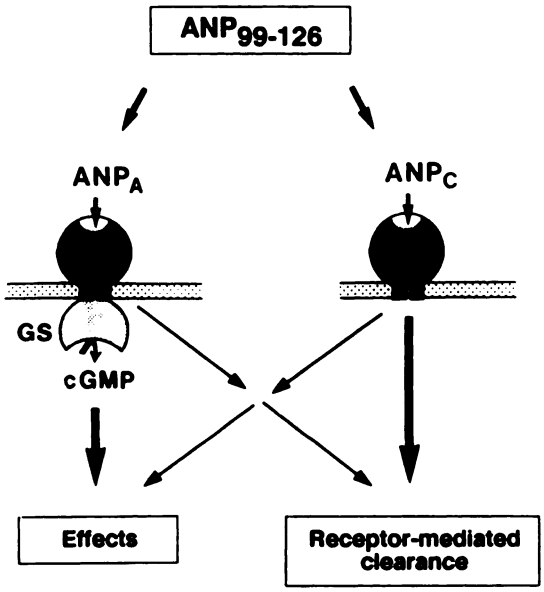

FIG. 8. ANP receptors and their major functions. Most studies suggest that a receptor that possesses guanylate cyclase activity mediates the renal actions of ANP, but the role of cGMP in mediating the effects of ANP on other tissues is less clear. Smaller arrows, possible biological effects of ANP_C receptor stimulation and internalization of ANP_A receptors.

about in half. The intracellular region contains a protein kinase-like domain and a carboxyl region known to be the cyclase domain (Thorpe and Morkin, 1990). The rat ANP_A receptor gene contains 22 exons spaced over a total of 17.5 kb pairs (Yamaguchi et al., 1990). ANP_{99-126} binds and stimulates ANP_A, ANP_B is not stimulated except at micromolar concentrations of ANP. BNP was more effective than ANP in binding to the ANP_B receptor and had increased guanylyl cyclase activity; however, relatively high concentrations were required, suggesting that the natural ligand is not either ANP or BNP (Chinkers and Garbers, 1991; Yuen and Garbers, 1992). Studies now show that CNP is the most selective endogenous ligand so far identified for the ANP_B receptor (Koller et al., 1991; Suga et al., 1992).

Results of most studies suggest that a receptor(s) that possesses guanylate cyclase activity mediates the renal actions of ANP (for reviews, see Waldman and Murad, 1987; Brenner et al., 1990; Cogan, 1990; Zeidel, 1990; Maack, 1992; Wilkins and Needleman, 1992). The primary sites of action of ANP in the nephron are the glomerulus and inner medullary collecting duct. The actions of ANP at these sites are accompanied by an increase in cGMP production and can be mimicked by dibutyryl-cGMP. On the other hand, the role of cGMP in mediating the effects of ANP on other tissues is less clear. ANP increases cGMP production in vascular smooth and endothelial cells. Yet, some analogs of ANP dissociate the cGMP-stimulating and -vasorelaxant actions of the peptide (Budzik et al., 1987). ANP receptor antagonists were also found to prevent guanylate cyclase activation but not hypotension in response to ANP (von Geldern et al., 1990). The effect of ANP on aldosterone secretion is not reproduced by 8-bromo-cGMP (Elliot and Goodfriend, 1986). ANP also inhibits aldosterone release independently of cGMP (Drewett et al., 1990, 1992; Johnson et al., 1991). The oxidized ANP analog, human [Met-O^{110}]ANP_{99-126}, at the doses that led to significant increase in diuresis, was neither natriuretic nor accompanied by an increase of urinary cGMP (Willenbrock et al., 1989), thus also dissociating the natriuretic and diuretic effects of ANP. Furthermore, Anand-Srivastava and coworkers (1984, 1987) reported that ANP inhibited basal and stimulated adenylate cyclase activity in numerous rat preparations by a pertussis toxin-sensitive mechanism. It also appears that ANP activates phospholipase C and increases inositol phosphate turnover and IP_3 (Resink et al,, 1988; Berl et al., 1991). These studies suggest that the ANP signal transduction mechanisms may not be limited to those involving guanylate cyclase, although one possibility is that the effects of ANP on these second messengers are secondary to the generation of cGMP (MacFarland et al., 1991; Jiang et al., 1992).

The ANP_C receptor is a truncated protein that binds ANP with high affinity and does not possess guanylyl cyclase activity (Fuller et al., 1988; Porter et al., 1989b, 1990). The binding affinity of human BNP for the human ANP_C receptor is about 1 order of magnitude lower than those of ANP; the rank order of binding affinity for the ANP_C receptor is ANP > CNP > BNP in both humans and rats (Suga et al., 1992). The human sequence is highly homologous (95%) to the bovine ANP_C receptor sequence and is expressed in placenta, kidney, and fetal heart (Porter et al., 1990). The bovine receptor is a 64-kDa transmembrane protein, which has a long extracellular ANP-binding domain (436 amino acids), a single hydrophobic transmembrane anchor (23 amino acids), and a very short, 37-residue, intracellular domain. The overall deduced structure of ANP_C receptor is similar to that of other receptors that are involved primarily in ligand sequestration and internalization, including those for low-density lipoproteins and insulin-like growth factor/mannose-6-phosphate. These proteins also contain a single transmembrane domain proximal to a short cytoplasmic tail. The extracellular domain of the ANP_C receptor shows homologies (30 to 48%) with ANP_A and ANP_B receptors, and the region that may be involved in ligand binding is >75% conserved between these receptors (Porter et al., 1990). The ANP_C receptor gene is localized to chromosome 5p13–14 (Chinkers and Garbers, 1991).

The ANP_C receptor is widely distributed in several tissues and cells, including kidney cortex, vascular, endothelial, and smooth muscle cells (Leitman and Murad, 1986; Leitman et al., 1986; Scarborough et al., 1986, 1988; Maack et al., 1987, 1988a; Olins et al., 1988; Martin et al., 1989; Nussenzveig et al., 1990b; Suga et al., 1992). In

these tissues and cells, the largest population of receptors are ANP_C receptors, which may make up >95% of the total ANP receptor population. Whereas ANP_A and ANP_B receptors display a high degree of selectivity for ANP_{99-126}, ANP_C receptor also binds fragments, internally ring-deleted ANP analogs, and some D-amino acid-substituted analogs with high affinity (Scarborough et al., 1988; Bovy et al., 1989). The structural feature responsible for recognition of the ANP_C receptor site appears to reside in a single sequence of seven (Gly^{108}–Gly^{114}; Bovy et al., 1989) or five (Arg^{109}–Ile^{113}; Okolicany et al., 1991) contiguous amino acids from the cyclic core of ANP. ANP analogs that specifically bind to ANP_C receptors increase circulating plasma IR-ANP levels (Maack et al., 1987). Nussenzveig et al. (1990b) showed that in cultured bovine aortic smooth muscle cells, in which ANP binds with high affinity to the large density of ANP_C receptors, the receptor-ligand complexes are rapidly internalized and the ligand is delivered to lysosomes where it undergoes complete hydrolysis to amino acids. ANP_C receptors are recycled to the cell surface membrane, maintaining the constant availability of receptors to mediate cycles of receptor-mediated endocytosis and lysosomal hydrolysis of ANP. These features of receptor-mediated internalization, lysosomal hydrolysis of ANP, and recycling of ANP_C receptors are qualitatively similar to those previously reported for other clearance receptors including low-density lipoproteins and asialoglycoproteins. Although a recent study (Rathinavelu and Isom, 1991) suggests that the ANP_A receptor also internalizes ANP, the high affinity and abundance of ANP_C receptors on vascular endothelial cells show that ANP_C receptors serve as powerful mechanisms to clear ANP from the circulation.

Recent findings suggest that some experimental manipulations inducing changes in blood volume and pressure may regulate the density of ANP_C-receptors. In view of the clearance function of C-receptors, one would expect that the decrease in ANP_C receptors would lead to increases in plasma IR-ANP concentration and vice versa. In support of this, water deprivation in rats was reported to decrease plasma IR-ANP levels and increase ANP_C receptor density on glomerular membranes (Kollenda et al., 1990), suggesting that both ANP release and elimination are involved in the control of plasma IR-ANP concentration and, thus, blood volume. The decrease in plasma IR-ANP levels following 5 weeks (but not 3 weeks) of an 8% NaCl diet was associated with an increased tissue uptake of ^{125}I-ANP from plasma, metabolic clearance rate, and V_{ds} compared with control rats (Widimsky et al., 1990b, 1991). Furthermore, $C\text{-}ANF_{4-23}$ infusion caused a significantly larger increase of plasma ^{125}I-ANP and a greater decrease in the metabolic rate and V_{ds} of ^{125}I-ANP in rats fed a high-concentration salt diet than in control animals (Widimsky et al., 1991). These results suggest that the increased availability of ANP_C receptors may be associated with the decrease in plasma IR-ANP levels and that physiological stimuli may regulate ANP_C receptors.

On the other hand, total glomerular ANP receptor density was down-regulated in response to 35 days of high salt intake in rats, and the decrease appeared to be due to a decrease in the number of ANP_C receptor sites; plasma IR-ANP levels remained unchanged (Michel et al., 1990b). Because the decreased number of ANP_C receptor-binding sites (decreased clearance) may result in a relatively greater amount of ANP binding to ANP_A receptors, normal plasma IR-ANP concentration may be sufficient to increase Na^+ excretion in response to salt load. Furthermore, Martin et al. (1989) showed increased ANP_C receptor density in renal glomeruli of SHR, and the density of glomerular C-type receptors was significantly increased in rats with ligated bile ducts at plasma IR-ANP concentrations not different from those of control rats (Gerbes et al., 1991). In vitro, vascular, or endothelial cell ANP_C receptors are not down-regulated by selective ligands (Cahill et al., 1990), but the increase in endothelial cell intracellular cGMP resulting from the activation of the guanylate cyclase of the ANP_A receptor may preferentially down-regulate ANP_C receptors (Kato et al., 1991; Rathinavelu and Isom, 1991) and inhibit the clearance of ANP vascular endothelial cells (Kato et al., 1992). ANP_C receptors on cultured endothelial cells are also sensitive to changes in the salt concentration of the incubation medium; exposure of the cells to NaCl resulted in a decrease in C-type receptors that was accompanied by a parallel reduction in the ANP_C receptor mRNA levels, and, interestingly, a marked potentiation of ANP-induced cGMP accumulation was observed (Katafuchi et al., 1992). Thus, although volume and pressure changes may affect ANP_C receptors, further studies of the regulation of ANP_C receptors under different physiological and pathophysiological conditions and of the effect of density of ANP_C receptors on the metabolism of ANP and circulating ANP levels are required.

There is some evidence that binding of ANP to ANP_C receptors elicits specific cellular responses, in addition to clearance of ANP from the circulation. It has been suggested that ANP_C receptors may mediate an inhibition of adenyl cyclase with a guanosine nucleotide-binding protein (Anand-Srivastava et al., 1990; Drewett et al., 1990). ANP_C receptor or a yet unidentified receptor with similar characteristics also mediates the neuromodulatory effect of ANP. Both ANP_{99-126} and des [Gln^{18}, Ser^{19}, Gly^{20}, Leu^{21}, Gly^{22}] $ANF_{4-23}\text{-}NH_2$ (called $C\text{-}ANF_{4-23}$), a peptide that binds to ANP_C receptors (Maack et al., 1987), inhibited evoked catecholamine release from differentiated pheochromocytoma cells (PC12 cells) (Drewett et al., 1990, 1992) and rabbit isolated vasa deferentia (Johnson et al., 1991). In these experiments, $C\text{-}ANF_{4-23}$ inhibited catecholamine release most likely via a pertussis toxin-sensitive inhibition of

adenylate cyclase without affecting cGMP generation. Anand-Srivastava et al. (1991) reported the presence of ANP_C receptors in rat platelets and the ability of ANP and C-ANF_{4-23} to attenuate cAMP generation in platelets by a pertussis-sensitive mechanism without any activation of cGMP. Hirata et al. (1989a) reported a stimulatory effect of the ANP_C receptor in activation of phosphoinositide hydrolysis in cultured bovine aortic smooth muscle cells using $ANP_{105-125}$ as a ligand. Cahill and Hassid (1991) reported that the antimitogenic effect of ANP in rat aortic smooth muscle cells may be mediated by a cGMP- and cAMP-independent mechanism via the ANP_C receptors. These reports suggest that the ANP_C receptor may be biologically active and also coupled to signal transduction system(s). Clearly, additional experiments are required to establish whether these effects are mediated by ANP_C receptors, ANP_C receptor subclasses, or other as yet unidentified ANP receptor(s) to which the ligands defined currently as selective ANP_C receptor ligands may bind.

2. Neutral endopeptidase. The renal handling of peptide hormones involves glomerular filtration followed by intraluminal or intracellular catabolism. The brush border of the proximal tubule is very rich in degradative enzymes and plays a major role in degrading many other peptides. ANP is also degraded by renal brush border membrane with concomitant loss of biological activity, as shown by several studies in which the metabolism of ANP was examined in vitro. Incubation of ANP_{99-126} with rabbit renal brush border membranes results in a single degradation product with a sequence of amino acids identical with that of native ANP_{99-126} but containing a cleaved peptide bond at the Cys^{105}-Phe^{106} (or Cys^{7}-Phe^{8}) within the 17-member ring leaving the disulfide bridge between Cys^{8}- and Cys^{23} intact (Olins et al., 1987a) (fig. 9). Porcine kidney microvillar membranes or rat kidney cortex membranes produce similar site-specific cleavages when incubated with ANP_{99-126} (Koehn et al., 1987; Stephenson and Kenny, 1987; Sonnenberg et al., 1988; Berg et al., 1988). Porcine membranes hydrolyzed ANP_{99-126} with a $t_{1/2}$ of 8 min (Stephenson and Kenny, 1987), and among subcellular fractions of rat kidney cortical homogenates, the luminal membranes were the most active in metabolizing ANP (Berg et al., 1988). The enzyme responsible for this cleavage is NEP, because phosphoramidon, a natural potent phosphoryl-containing inhibitor, or other NEP inhibitors caused suppression of the products and other peptidase inhibitors had no significant effect (Stephenson and Kenny, 1987, 1988; Bertrand and Doble, 1988; Kenny and Stephenson, 1988; Sonnenberg et al., 1988; Vanneste et al., 1988; Olins et al., 1989).

NEP is a metallopeptidase with zinc in its active center; it cleaves the α-amino bond of hydrophobic amino acids, provided that the susceptible bond does not involve the COOH- or NH_2-terminal amino acid (for a review,

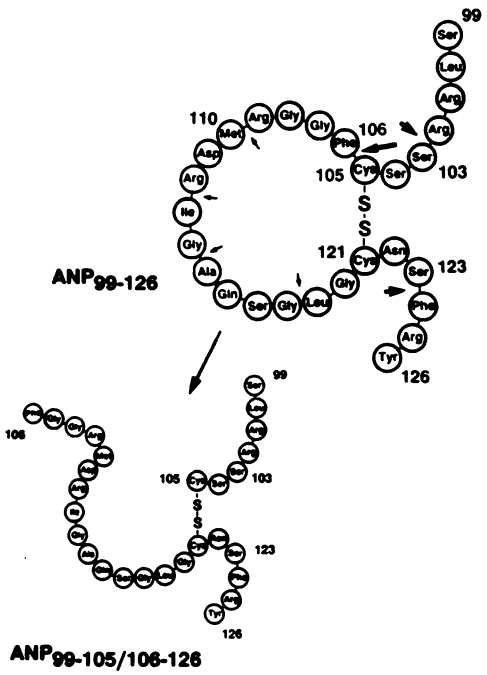

FIG. 9. Enzymatic degradation of ANP (according to Olins et al., 1987b; Stephenson and Kenny, 1987; and Johnson et al., 1989).

see Erdös and Skidgel, 1989). The cDNA for human, rat, and rabbit NEP has been cloned and reveals a high degree of homology (93 to 94%). In tissues and cells, NEP is bound to the plasma membrane through a hydrophobic membrane-spanning domain near the NH_2 terminus and is present in a soluble form in plasma and urine. In addition to ANP, NEP metabolizes numerous vasoactive peptides, including enkephalins, bradykinin, substance P, neurotensin, gastrin, and chemotactic peptide. Although the kidney has the greatest capacity for peptide degradation, the renal brush border is not the only site of ANP degradation by NEP; the endopeptidase is also distributed in brain, lung, thyroid, parts of the intestine, seminal vesicles, and prostate (Erdös and Skidgel, 1989), as well as in plasma (Yandle et al., 1989) and vascular tissue (Tamburini et al., 1989). The endopeptidase also appears to hydrolyze BNP in renal microvilli and choroid plexus membranes (Vogt-Schaden et al., 1989; Bourne and Kenny, 1990; Vanneste et al., 1990a).

Prolonged incubation with NEP further degrades ANP_{99-126} to smaller fragments, resulting from the hydrolysis at seven sites including Ser^{123}-Phe^{124} bond (fig. 9) (Stephenson and Kenny, 1987; Chondra et al., 1988; Vanneste et al., 1988). The rate-limiting cleavage in the inactivation of the peptide is at the Cys^{105}-Phe^{106} peptide bond. Generation of the ring-opened metabolites ANP_{99-126}, $ANP_{103-126}$, or $ANP_{103-123}$ by NEP results in loss of biological activity (Olins et al., 1987a,b; Seymour et al., 1988). However, the cleaved $ANP_{99-105/106-126}$ was recently reported to stimulate guanylate cyclase and to alter hemodynamics and the renin-ANG system in vivo in conscious sheep. This could be explained by binding of the cleaved product to ANP_C receptors, but method-

ology made it difficult to measure the increase in intact IR-ANP concentration in plasma during infusion of the cleaved peptide (Charles et al., 1991b). One of the early findings suggesting that endopeptidase may degrade ANP in vivo was the observation that 20 to 30% of the total ANP present in human coronary sinus plasma made up a peptide corresponding to $ANP_{106-126}$ (Yandle et al., 1987). Incubation of the 24-amino acid peptide, $ANP_{103-126}$ (atriopeptin III), the amino-terminal truncated metabolite of ANP_{99-126}, with renal brush border membranes also resulted in the cleavage at the Ser^{103}-Ser^{104}, Cys^{105}-Phe^{106}, and Ser^{123}-Phe^{124} bonds (Koehn et al., 1987; Olins et al., 1987a). Cleavage of $ANP_{103-126}$ at Ser^{103}-Ser^{104} to release the NH_2-terminal serine may be due to aminopeptidase activity (Olins et al., 1987a).

3. Other inactivation pathways. a. ANGIOTENSIN-CONVERTING ENZYME. In addition to the NEP- and receptor-mediated clearance of ANP, several reports have suggested other enzymes in the metabolism of ANP, including ACE and kallikrein. Early in vitro studies suggested that ACE may be involved in the metabolism of ANP, because SQ 20881 inhibited the degradation of $ANP_{103-126}$ in kidney homogenates (Tang et al., 1984b). Consistent with this study, in anesthetized rats, a role for ACE in metabolism of ANP was suggested by the observation that ACE inhibition potentiated the natriuretic activity of the ANP contained in extracts of monkey atria (Wang and Gilmore, 1985). $ANP_{103-125}$ has been reported to be hydrolyzed by cardiac converting enzyme to $ANP_{103-123}$ in vitro (Sakharov et al., 1988).

In vivo experiments in normal animals have shown either enhancement, no change, or even a decrease in ANP-induced biological effects after ACE inhibition (di Nicolantonio et al., 1986; Hansell and Ulfendahl, 1987; Di Nicolantonio and Morgan, 1987; Seymour et al., 1989b; Smits et al., 1990a; Chamienia and Johns, 1992). Studies in normal humans have shown that ACE inhibition increases (Wilkins et al., 1987), has no effect (Gaillard et al., 1989; Wambach et al., 1989), or decreases (Mann et al., 1986) endogenous plasma IR-ANP levels, although a decrease in ANP-induced natriuresis during ANP inhibition in normal humans has been a consistent finding (Wilkins et al., 1987; Gaillard et al., 1989; Wambach et al., 1989). However, because ACE inhibitors affect hemodynamics and renal blood flow, which influence the biological response to ANP, the interpretation of changes in ANP bioactivity during ACE inhibition is difficult. Studies by Stephenson and Kenny (1987) showed that captopril had no effect on the hydrolysis of ANP_{99-126} by pig kidney microvillar membranes. In humans, endogenous plasma levels, metabolic clearance, and biological effects of ANP were not altered by acute inhibition of converting enzyme during low-dose infusion of ANP (Richards et al., 1989b). These results suggest that converting enzyme does not play a major role in the metabolism of ANP and that the observed changes in plasma IR-ANP concentration and ANP bioactivity reflect changes in hemodynamic variables and volume status during acute and chronic ACE inhibition.

b. KALLIKREINS. Several early findings suggested that kallikrein may also be involved in the degradation of ANP. A 1-hour incubation with kallikrein significantly reduced the natriuretic activity of extracts of rat atrial tissue (Briggs et al., 1984). Aprotinin, an inhibitor of serine proteases, enhanced the natriuretic responses to atrial extracts (Briggs et al., 1984) and prolonged the renal effects of $ANP_{101-126}$ (Thibault et al., 1984). In anesthetized rats, $ANP_{101-126}$ elevated urinary kallikrein excretion (Garcia et al., 1985d). Furthermore, glandular kallikrein diminished the in vitro vasorelaxant activity of $ANP_{103-126}$ in a time-dependent manner (de Mey et al., 1987). However, aprotinin did not enhance the depressor activity of exogenous ANP_{99-126} in conscious SHR, suggesting that inactivation of ANP does not solely depend on kallikrein (Seymour et al., 1989b). De Mey and colleagues (1987) also concluded that kallikrein was not present in sufficient amounts in renal microsomes to account for the observed rate of ANP degradation by that preparation. In contrast, in a recent study, aprotinin increased the $t_{1/2}$ of infused iodinated ANP from 0.4 to 2.4 min in anesthetized rats, suggesting that a kallikrein-like activity is physiologically involved in the metabolism of the peptide (Vanneste et al., 1990b). Furthermore, amino acid analysis of ANP fragments produced by purified human kallikrein led to the identification of eight cleavage sites within the peptide sequence, and cleaved Cys^{105}-Phe^{106} ANP disappeared 4-fold faster than ANP_{99-126} under kallikrein action (Vanneste et al. 1991). A maximal inhibition of ANP degradation was observed when both aprotinin and phosphoramidon were given simultaneously, the $t_{1/2}$ of ANP increasing from 0.4 to 15 min (Vanneste et al., 1990b). This cumulative effect of the NEP and the kallikrein inhibitors in the protection of the circulating peptides suggests that both enzymes may play a part in the clearance and inactivation of ANP.

c. OTHER POSSIBLE ATRIAL NATRIURETIC PEPTIDE-DEGRADING ENZYMES. As described before, experiments in which cell cultures were used indicate that receptor-bound ANP is endocytosed and delivered to the lysosomes (Hirata et al., 1985; Napier et al., 1986; Pandey et al., 1987) and that the predominant binding site in cultured vascular smooth muscle is thought to act as a clearance receptor. Recently, Johnson and colleagues (Johnson et al., 1989; Johnson and Foster, 1990) reported the presence of a metalloendopeptidase in vascular smooth muscle and endothelial cells that was not inhibited by NEP inhibitors (SCH 39370, phosphoramidon) or captopril and that cleaved the COOH-terminal tripeptide from ANP. Because it is generally believed that the COOH-terminal tripeptide is important for many of ANP's bioactivities, this enzyme may be involved in the inactivation of ANP in target tissues. New atrial peptide-

degrading enzymes from bovine kidney (Toll et al., 1991) and human neuroblastoma NB-OK-1 cells (Delporte et al., 1992), which also displays high affinity and selectivity for the Ser[123]-Phe[124] bond, appear to be similar to the metalloendopeptidase described by Johnson and his coworkers.

Initial chemical isolation studies showed that incubation of the atrial extracts with proteinases, including trypsin, abolished their biological activity (de Bold et al., 1982a,b; Garcia et al., 1982; Trippodo et al., 1983; Briggs et al., 1984). Carboxypeptidase B, α-chymotrypsin, and elastase were also reported to reduce the biological activity of atrial extracts (Thibault et al., 1983; Briggs et al., 1984). Bestatin, an inhibitor of aminopeptidase, slowed the disappearance of $ANP_{103-126}$ (Tang et al., 1984b), and amastatin, another aminopeptidase inhibitor, had no effect on the hydrolysis of ANP_{99-126} by pig kidney microvillar membranes (Stephenson and Kenny, 1987). In brain, metalloendopeptidase insensitive to phosphoramidon was capable of degrading ANP in vitro (Bourne and Kenny, 1990). A soluble metalloendopeptidase contributes to the ANP degradative activity found in ventricular muscle preparations (Rugg et al., 1988). Finally, a membrane-bound metalloendopeptidase from rat kidney was recently reported to hydrolyze ANP at Ser[117]-Gly[118] and Asn[122]-Ser[123] bonds (Yamaguchi et al., 1992). The physiological importance of these peptidases remains to be clarified.

d. ROLE OF PLASMA AND ELIMINATION IN URINE. Veress et al. (1985) reported that blood, especially a white blood cell/platelet fraction, reduced the natriuretic activity of the incubation media containing released ANP. However, blood and plasma degradation in vitro play only a minor role in ANP elimination (Murthy et al., 1986a; Almeida et al., 1989; Gros et al., 1990a). ANP_{99-126} is converted very slowly (about 10 to 15% in 30 min) by a plasma protease into a 24-amino acid form, $ANP_{103-126}$ (Murthy et al., 1986a). This would probably account for the presence of $ANP_{103-126}$ as a minor form in the circulation of the rat (Schwartz et al., 1985). No $ANP_{103-126}$ could be measured in the urine in anesthetized rats (Luft et al., 1986a) or in humans (Gnädinger et al., 1986). The very low urinary radioactivity after injection of the iodinated peptide (Widimsky et al., 1990b), which could also include cleavage products, supports the notion that urinary excretion does not play a major role in ANP elimination under normal physiological conditions. Thus, NEP is capable of degrading any ANP that is normally filtered by the kidney. However, inhibition of NEP results in appearance of large amounts of ANP in the urine even in the rat (see section V.O.2).

D. Inhibitors of Atrial Natriuretic Peptide Elimination

1. ANP_C receptor ligands. ANP_C receptors bind not only native ANP_{99-126} but also several synthetic peptide analogs of ANP with deletions of amino acids in the COOH-terminal and ring-deleted peptide analogs, including C-ANF_{4-23} (Leitman et al., 1986; Leitman and Murad, 1986; Scarborough et al., 1986; Maack et al., 1987) and SC 46542 [des(Phe[106], Gly[107], Ala[115], Gln[116])-$ANP_{103-126}$](Koepke et al., 1989, 1990a,b) with high affinity. These molecules were shown to lose their renal activities in vitro but retain high-affinity binding to ANP receptors in cultured endothelial and vascular smooth muscle cells. In the isolated perfused rat kidney preparation, C-ANF_{4-23} displaced >95% of ^{125}I-ANP_{99-126} from its binding sites in whole kidney and kidney cortex at a perfusate concentration of 100 nM (Maack et al., 1987). Furthermore, excess perfusate concentrations of C-ANF_{4-23} (1 μM) were devoid of detectable renal effects and did not antagonize any of the known renal and hemodynamic actions of ANP_{99-126} in the isolated perfused rat kidney, including GFR, filtration fraction, renal vascular resistance, and fluid and electrolyte excretion. Thus, C-ANF_{4-23} had no agonist or antagonist action, in spite of occupying >95% of the total ANP receptor sites in the isolated perfused rat kidney (Maack et al., 1987). In contrast, in vivo C-ANF_{4-23} increased sodium excretion and tended to decrease blood pressure in anesthetized rats with an increase in plasma IR-ANP levels (Maack et al., 1987). These results showed that it is possible to increase plasma IR-ANP levels by decreasing its binding to ANP_C receptors with ligands that bind to ANP_C receptors but not to ANP_A receptors and that ANP_C receptors may be involved in the metabolic clearance of ANP.

a. EFFECTS OF ANP_C RECEPTOR LIGANDS ON EXOGENOUS AND ENDOGENOUS ATRIAL NATRIURETIC PEPTIDE. Several lines of evidence show that ANP_C receptors have an important clearance function in intact animals. Administration of C-ANF_{4-23} (1 and 10 μg/kg/min) altered the pharmacokinetics of a single bolus injection of ^{125}I-ANP_{99-126} in anesthetized rats by reducing the apparent V_{ds} and metabolic clearance rate at steady state (Almeida et al., 1989). At the highest dose, C-ANF_{4-23} decreased V_{ds} from 97 to 36 ml/100 g body weight and metabolic clearance rate from 59 to 12 ml/min/100 g body weight, whereas plasma $t_{1/2}$ of ^{125}I-ANP_{99-126} increased from 1.3 to 2.2 min. Consistent with the role of C-type receptors in the metabolism of ANP, blockade of ANP_C receptors also led to a marked delay in the appearance of ^{125}I-ANP_{99-126} hydrolytic products in plasma (Almeida et al., 1989). Chiu et al. (1991) generated similar data concerning the pharmacokinetics of ^{125}I-ANP in anesthetized rats, whereas C-ANF_{4-23} did not affect ANP clearance in early third-trimester fetal sheep (Castro et al., 1991). Furthermore, SC 46542 was as potent as ANP_{99-126} in inhibiting ^{125}I-ANP_{99-126} binding to mouse lung membranes in vivo (Souque et al., 1992). The profile of action of ANP_C receptor ligands on the pharmacokinetics of ANP differs from that of inhibitors of NEP; ANP_C receptor ligands markedly reduced the steady-state V_{ds}, delayed clearance,

and prolonged the $t_{1/2}$ of ANP, whereas NEP inhibitors predominantly affected the clearance of the peptide (Chiu et al., 1991).

C-ANF$_{4-23}$ infusion also has been shown to increase the plasma concentration of endogenous IR-ANP in anesthetized and conscious rats (Maack et al., 1987; 1988b). Constant infusion of C-ANF$_{4-23}$ (1 μg/kg/min) increased plasma levels of IR-ANP in anesthetized rats from 51 to 141 pg/ml and in conscious rats from 72 to 131 pg/ml. In most rats, infusion of C-ANF$_{4-23}$ approximately doubled plasma IR-ANP concentration. Two linear polypeptides containing only 10 (Ala^7-ratANP$_{8-17}$-NH$_2$) or five (2-naphtoxyacetyl-isonipecotyl-Arg-Ile-Asp-Arg-Ile-NH$_2$) amino acids have also been shown to increase plasma IR-ANP levels in anesthetized rats (Okolicany et al., 1991). Similarly, i.v. administration of C-ANF$_{4-23}$ (1 μg/kg/min for 60 min) caused 2- to 3-fold elevations of endogenous ANP levels in both hypoxia-adapted and air-breathing control rats (Jin et al., 1990). In conscious sheep, plasma IR-ANP concentration increased from 28 to 85 pg/ml after 80 min of infusion (400 μg/h) of C-ANF$_{4-23}$ (Parkes et al., 1990), whereas C-ANF$_{4-23}$ infusion into fetal sheep significantly increased fetal plasma IR-ANP concentration, and the combined C-ANF$_{4-23}$/ANP$_{99-126}$ produced a further increase in fetal IR-ANP concentrations (Castro et al., 1991). Interestingly, rat BNP$_{1-45}$ (iso-rANP) also increased plasma IR-ANP concentrations, probably by displacing ANP from ANP$_C$ receptors (Jennings et al., 1990). In a recent study, infusion of C-ANF$_{4-23}$ for 30 min elevated basal plasma IR-ANP levels moderately in conscious normal rats (Kukkonen et al., 1992). IR-NT-ANP remained unchanged after C-ANF$_{4-23}$ infusion, showing that treatment did not influence the basal release of the peptide from the heart. These findings are consistent with a major function of ANP$_C$ receptors in the metabolic clearance of ANP and that plasma IR-ANP concentration is determined by the C-receptor system. Yet, others have reported that C-ANF$_{4-23}$ in the conscious normal and DOCA/salt hypertensive rat (Seymour et al., 1991b) and SC 46542 in conscious normal rats (Koepke et al., 1989) or SHR (Koepke et al., 1990a,b) do not significantly increase plasma IR-ANP concentration.

The role of ANP$_C$ receptors in the regulation of plasma IR-ANP concentration has been further studied during elevated endogenous plasma IR-ANP levels induced by atrial stretch. Interestingly, the elevations of plasma IR-ANP concentrations by volume loading were not enhanced by infusion of C-ANF$_{4-23}$. The relation between the change in plasma IR-ANP concentration and the change in right atrial pressure did not shift to the left, and thus, for a given degree of atrial stretch, plasma IR-ANP concentration was similar in C-ANF$_{4-23}$-treated rats to that in controls (Kukkonen et al., 1992). Why ANP$_C$ receptor ligands appear to increase basal plasma IR-ANP concentrations but failed to enhance elevated plasma IR-ANP levels induced by volume loading in conscious rats remains to be established. C-ANF$_{4-23}$ at the concentrations used did not directly inhibit stretch-stimulated ANP release from the heart when the release of endogenous ANP was assessed by IR-NT-ANP measurements (Kukkonen et al., 1992). These data suggest that C-ANF$_{4-23}$-induced elevation in plasma IR-ANP concentration may be buffered by inactivation of ANP by other elimination pathways including NEP involved in the removal of ANP from the circulation, thus offsetting the further enhancement of peptide levels induced by volume load.

b. RENAL AND HEMODYNAMIC EFFECTS OF ANP$_C$ RECEPTOR LIGANDS. The increase in plasma ANP caused by C-ANF$_{4-23}$ in anesthetized rats has been reported to be accompanied by expected renal and hemodynamic effects (Maack et al., 1987, 1988b). Mean blood pressure decreased slightly (by 9 mm Hg), and there were significant increases in GFR, urine volume, and sodium excretion. These changes corresponded to those caused by an infusion of ANP$_{99-126}$, which increased plasma IR-ANP concentration to the same extent as that following the infusion of C-ANF$_{4-23}$. The increase in plasma IR-ANP concentration in response to infusion of Ala^7-ratANP$_{8-17}$-NH$_2$ (10 amino acids) and 2-naphtoxyacetyl-isonipecotyl-ratANP$_{11-14}$-NH$_2$ (five amino acids) or linear fragments from the ring structure of ANP$_{99-126}$ significantly decreased blood pressure and increased GFR and sodium excretion in anesthetized rats (Okolicany et al., 1991). In conscious sheep, infusion of C-ANF$_{4-23}$ (400 μg/h) reduced blood pressure, cardiac output, and stroke volume and slightly increased peripheral resistance (Parkes et al., 1990). These hemodynamic changes were similar to those observed with ANP$_{99-126}$ infusion in sheep (Charles et al., 1990). Furthermore, the duration of hypotensive action from injection of ANP$_{99-126}$ was increased 2-fold during infusion of C-ANF$_{4-23}$ (Parkes et al., 1990). In fetal sheep, C-ANF$_{4-23}$ significantly increased natriuresis and diuresis, and these effects were enhanced by combined ANP$_{99-126}$ infusion (Castro et al., 1991). Similarly, another ANP$_C$ receptor ligand, SC 46542, potentiated the depressor response to low-dose infusion of ANP$_{103-126}$ (Koepke et al., 1989) but produced only a slight increase in sodium excretion and urine volume in conscious rats (Koepke et al., 1989) or no change in SHR and WKY rats (Koepke et al., 1990b). C-ANF$_{4-23}$ (1 μg/kg/min for 60 min) decreased blood pressure (Seymour et al., 1991b) and at a higher dose (100 μg/kg i.v. bolus plus 10 μg/kg/min for 30 min) significantly reduced blood pressure and increased plasma cGMP concentration as well as urinary excretion of cGMP, sodium, and water in conscious DOCA/salt hypertensive rats (Vemulapalli et al., 1991). C-ANF$_{4-23}$ also significantly lowered mean pulmonary arterial pressure in hypoxia-adapted rats but not in air-breathing controls, whereas systemic arterial pressure did not

change significantly in either group (Jin et al., 1990). However, C-ANF$_{4-23}$ and SC 46542, when administered alone, had no effect on hemodynamic parameters in normal conscious rats (Koepke et al., 1989, 1990b; Seymour et al., 1991b; Kukkonen et al., 1992). Furthermore, chronic infusion of SC 46542 for 10 days had no effect on mean arterial pressure in SHR, and no significant interaction was found between the SC 46542 and ANP$_{103-126}$ (Koepke et al., 1990a).

In summary, the acute influence of ANP$_C$ receptor ligands on plasma IR-ANP concentration as well as on cardiovascular and renal function has been assessed in some experimental models of pressure and volume overload and in normal rats and sheeps. The results show that, in addition to the release of ANP from the heart, a receptor-mediated clearance of ANP appears to regulate the circulating levels of ANP. However, the enhancement of plasma IR-ANP concentration by ANP$_C$ receptor ligands may be limited or undetectable under some experimental conditions. Data from low-dose infusions of ANP in humans and animals (in which changes in plasma IR-ANP concentrations may be small, see section VI.A) suggest that the range of significant biological effects documented in these studies may be attributable to the observed increase in plasma IR-ANP concentration. At present, there are no published data of the effects of ANP$_C$ receptor ligands in humans. Because the structural requirements are far less stringent than those for binding to ANP$_A$ receptors, ANP$_C$ receptor ligands may be potentially useful for increasing plasma levels of endogenous ANP.

2. Neutral endopeptidase inhibitors. Originally, inhibitors of NEP, such as thiorphan and phosphoramidon, were designed to prevent the degradation of enkephalins in brain tissue and were studied for their analgesic properties (Schwartz et al., 1990). The finding that ANP is a substrate for NEP has stimulated reassessment of these older agents as well as development of new specific inhibitors of the enzyme. New potent inhibitors of NEP have been reported, and all agents have provided useful tools with which to investigate the role of NEP in the inactivation of ANP (for reviews, see Gerbes and Vollmar, 1990; Roques and Beaumont, 1990; Schwartz et al., 1990; Sybertz, 1991).

Commonly, specific NEP inhibitors exert ANP-like effects by competitively inhibiting its metabolism in vitro and in vivo. NEP inhibitors stereochemically resemble ACE inhibitors, and the rational design and synthesis of potent and specific inhibitors for endopeptidase 24.11 has been in progress for several years using a simplified model of the active site of other metalloproteases, such as carboxypeptidase A, thermolysin, and ACE. Various functional groups have been introduced on di- or tripeptide-like structures that will interact with the hydrophobic pocket of the enzyme, and four different classes are now available: thiol, carboxyl, phosphoryl, and hydroxamate inhibitors (Gerbes and Vollmar, 1990; Roques and Beaumont, 1990; Schwartz et al., 1990; Sybertz, 1991). Thiol inhibitors are represented by thiorphan, the first synthetic inhibitor of endopeptidase (Gros et al., 1989; Trapani et al., 1989), and SQ 29072, a selective NEP inhibitor (Seymour et al., 1989a,b). SCH 39370 (Sybertz et al., 1989) and UK 69578 (Danilewizc et al., 1989) are carboxyl inhibitors that contain a hydrophobic moiety, significantly improving the affinity for endopeptidase. Phosphoryl and hydroxamate inhibitors including kelatorphan contain a strong Zn-interacting group. The new specific compounds inhibit purified kidney NEP at concentrations in the low nanomolar range and are devoid of activity on ACE or other peptidases, such as carboxypeptidase A. For example, UK 69578 inhibits endopeptidase activity in a competitive manner with a K_i of 5.2 $\times 10^{-8}$ M (Northridge et al., 1989). At concentrations up to 10^{-4} M, UK 69578 had no significant effect on ACE, carboxypeptidase A, leucine aminopeptidase, trypsin, chymotrypsin, or renin. Some NEP inhibitors are prodrugs, which after hydrolysis release the active endopeptidase inhibitors into the circulation. Thus, with the availability of these oral prodrugs, controlled studies are in progress to evaluate the NEP inhibitors as potential diuretic and antihypertensive agents in humans. The profile of action of NEP inhibitors as ANP-like compounds, emerged with the different drugs, is shown in table 4 and will be summarized below.

a. EFFECTS OF NEUTRAL ENDOPEPTIDASE INHIBITORS ON EXOGENOUS ATRIAL NATRIURETIC PEPTIDE. The influence of NEP inhibitors in response to administration of pharmacological doses of ANP has been assessed in a variety of experimental models. For example, NEP inhibitors SQ 29072 (30 to 100 μmol/kg, i.v.) (Seymour et al., 1989a,b) and SCH 34826 (10 to 90 mg/kg, orally) (Sybertz et al., 1990a) significantly potentiated the depressor, diuretic, and natriuretic actions and urinary cGMP responses of ANP in spontaneously hypertensive and DOCA/salt rats, respectively. Consistent with inhi-

TABLE 4
Some pharmacological properties of NEP inhibitors

Prevention of degradation of ANP in vitro and in vivo
Reduction of clearance of ANP (and prolongation of $t_{1/2}$)
Enhancement of biological actions of ANP
Potentiation of diuretic, natriuretic, hypotensive, and plasma ANP responses to administration of pharmacological doses of exogenous ANP
Potentiation of the natriuretic and diuretic response to volume expansion
Acute decrease in blood pressure in volume-dependent models of hypertension (DOCA/salt hypertensive rat)
Production of diuresis, natriuresis, and an increase in plasma ANP levels in CHF models or in rats with reduced renal mass with enhanced cGMP and ANP excretion
Diuresis, natriuresis, and a modest change in plasma ANP levels in human volunteers and patients with CHF
Variable or no responses in the normotensive models or after short-term administration

bition of ANP degradation, NEP inhibitors caused a marked elevation of the plasma levels of IR-ANP subsequent to injection of large doses of ANP. UK 69578 (3 mg/kg, i.v.) extended the $t_{1/2}$ of exogenous ANP (200-ng bolus of rat ANP) from 0.72 to 1.0 min in normal and from 0.96 to 2.5 min in nephrectomized rats and increased the natriuretic and diuretic responses in anesthetized rats (Northridge et al., 1989). Similar results in normal or hypertensive animals with other NEP inhibitors such as thiorphan (Olins et al., 1989; Trapani et al. 1989; Webb et al., 1989; Smits et al., 1990a), phoshoramidon (Webb et al., 1989), (S,S)-HCBA (Olins et al., 1989), SQ 28603 (Gardiner et al., 1992), and SCH 39370 and derivatives (Haslanger et al., 1989; Sybertz et al., 1989, 1990b, 1991; Charles et al., 1991c) have been reported. Furthermore, NEP inhibition abolished the clearance and extraction of plasma ANP across the lung, prevented the decrease in the urinary clearance of ANP, and enhanced the renal response to endogenous ANP in dogs with acute experimental CHF (Perrella et al., 1991b). These results show that NEP plays an important role in determining the metabolic fate of ANP.

To determine whether NEP inhibitors interfered with metabolism of physiological levels of ANP, the effects on the pharmacokinetics of i.v. injections of tracer quantities of radiolabeled ANP have been assessed. Generally, trichloroacetic acid-precipitable radioactivity, reflecting intact ANP, declines rapidly after an i.v. bolus injection of ANP. Concomitantly, trichloroacetic acid-soluble radioactivity increases. Antihypertensive doses of SCH 34826 (90 mg/kg, subcutaneously) (Chiu et al., 1991) or SCH 39370 (30 mg/kg, subcutaneously) (Sybertz et al., 1990b) significantly delayed the disappearance of intact peptide from the circulation, inhibited the appearance of degradation products in the plasma, and prolonged plasma $t_{1/2}$ of ANP in the DOCA/salt rat, indicating that inhibition of NEP has a significant effect on the disposition of tracer quantities of ANP. In anesthetized rats, the plasma clearance of ^{125}I-ANP persistently showed a moderate decrease (about 40%), whereas $t_{1/2}$ values did not significantly differ from controls (Chiu et al., 1991). Candoxatrilat (UK 69578; 3 mg/kg, i.v.) reduced the clearance and the elimination $t_{1/2}$ of ^{125}I-ANP and ANP_{5-126}, whereas V_{ds} remained unchanged; in nephrectomized rats, terminal elimination $t_{1/2}$ was doubled (Barclay et al., 1991). In conscious rabbits, thiorphan induced a 32% reduction in the systemic clearance of ^{125}I-ANP, whereas $t_{1/2}$ and V_{ds} remained unchanged (Marleau et al., 1991). In mice, the degradation of ^{125}I-labeled ANP was markedly delayed by acetorphan, thiorphan, sinorphan, and retorphan, as shown by the levels of the intact peptide in the plasma or kidney (Gros et al., 1989, 1990a,b; Bralet et al., 1991; Souque et al., 1992). Results of these studies indicate that the metabolic transformation of ^{125}I-labeled ANP to its final breakdown product, iodotyrosine, is blocked by NEP inhibitors and that these interfere with ANP metabolism.

b. EFFECTS OF NEUTRAL ENDOPEPTIDASE INHIBITORS ON ENDOGENOUS ATRIAL NATRIURETIC PEPTIDE. Experiments have shown some inconsistency in the ability of NEP inhibitors to increase plasma IR-ANP levels in vivo. In general, the failure of NEP inhibitors to elevate plasma IR-ANP levels has been found in the normal intact rat, whereas acute administration of NEP inhibitors increase plasma IR-ANP concentration in different experimental models with elevated endogenous plasma IR-ANP levels. NEP inhibitors have been shown to increase plasma IR-ANP levels in DOCA/salt hypertensive rats; Sybertz et al., 1990a; Seymour et al., 1991b; Shepperson et al., 1991), in NaCl-sensitive SHR (Jin et al., 1992), in rats with reduced renal mass (5/6 nephrectomy) (Lafferty et al., 1989), in rat CHF models (Tikkanen et al., 1990; Wilkins et al., 1990a; Helin et al., 1991), in dogs after 8 days of rapid right ventricular pacing (Cavero et al., 1990), in cardiomyopathic hamsters with CHF (Smits et al., 1990b), and in ovine heart failure induced by rapid ventricular pacing (Fitzpatrick et al., 1992). When given to dogs with surgically induced atrioventricular block as a model of chronic heart failure, UK 69578 (3 mg/kg) doubled plasma IR-ANP concentrations with a concomitant increase in urinary sodium excretion (Northridge et al., 1989). In rats with experimental chronic heart failure, NEP inhibition by SCH 39370 elevated plasma IR-ANP and cGMP concentrations for 6 days (Helin et al., 1991). NEP inhibitors have also been reported to elevate plasma IR-ANP levels when endogenous ANP was increased by volume loading (Danilewicz et al., 1989; Northridge et al., 1989; Schwartz et al., 1990; Bralet et al., 1991; Kukkonen et al., 1992). Kukkonen et al. (1992) compared the effect of atrial stretch induced by volume expansion on plasma ANP concentrations in vehicle- and the SCH 39370-treated animals and found that the relationship between changes in plasma IR-ANP concentration and right atrial pressure shifted to the left; thus, for a given degree of atrial stretch, plasma IR-ANP concentration was higher in SCH 39370-treated rats than in controls. These results support the conclusion that NEP is an important pathway for the inactivation of endogenous ANP.

In contrast, in anesthetized and conscious normal rats (Lafferty et al., 1989; Koepke et al., 1989; Olins et al. 1989; Sybertz et al., 1989; Trapani et al., 1989; Smits et al., 1990a; Tikkanen et al., 1990; Wilkins et al., 1990a; Seymour et al., 1991b), hamsters (Smits et al., 1990b), and monkeys (Seymour et al., 1991a) and also in DOCA/salt hypertensive rats (Sybertz et al., 1989, 1990a,b, 1991) and SHR (Koepke et al., 1990b), various NEP inhibitors have been reported to be ineffective or only to increase plasma IR-ANP concentrations when pharmacological concentrations of ANP were concomitantly administered (Olins et al., 1989; Trapani et al., 1989). Yet, others have

reported that NEP inhibitors increase plasma IR-ANP concentration in normal anesthetized dogs (Margulies et al., 1990), normal rabbits (Marleau et al., 1990, 1991), normal sheep (Charles et al., 1991c), and humans (Gros et al., 1989; Richards et al., 1990). Recently, Kukkonen et al. (1992) noted a dose-dependent elevation in IR-ANP by SCH 39370 in chronically cannulated normotensive conscious rats. These discrepant findings could be due to the differences in the NEP inhibitor used or to differences in experimental design. For example, changes in plasma ANP may be evident only soon after administration of NEP inhibitors. In support of this hypothesis, in DOCA/salt hypertensive rats, plasma ANP was significantly elevated by SCH 34826 when measurements were made 1, but not 3, h after giving the drug (Sybertz et al., 1990a). Furthermore, it is possible that the extensive binding of ANP to membrane surfaces, activation of other elimination pathways for ANP, or decreased release of ANP as result of a reduction in atrial distension may mask an influence of NEP inhibition on the steady-state plasma IR-ANP levels. These processes may become insignificant in animals treated with pharmacological doses of the exogenous peptide or when pressure and overload induce high baseline levels of ANP. Thus, high ANP levels and NEP activity may be necessary for enzyme inhibition to significantly augment plasma ANP levels and have physiological effects. The regulation of plasma ANP levels may also be more dependent on endopeptidase under conditions of elevated plasma ANP levels because of reduced capacity to clear circulating ANP via a receptor-mediated mechanism. Under such conditions, endopeptidase inhibition should result in increased circulating ANP levels in heart failure but not in normal animals in which all ANP clearance mechanisms are fully operative.

Although the above-mentioned drugs are selective inhibitors of NEP, one possible explanation for their ability to increase plasma ANP would be via the release of ANP from atria. In a study by Singer et al. (1991) of hypertensive patients, levels of IR-NT-ANP were significantly higher when patients received a high sodium diet rather than a low sodium diet, and there was no significant change in the plasma NT-ANP concentration with NEP inhibition in the 6 h after a single dose of candoxatril. Kukkonen et al. (1992) also measured the plasma immunoreactivity of the NH_2-terminal fragment of the prohormone of ANP to characterize the endogenous ANP secretion during SCH 39370 administration. The baseline IR-NT-ANP levels were higher than ANP levels and were not changed after vehicle or SCH 39370 administration. Moreover, analysis of changes in plasma IR-ANP concentration against change in right atrial pressure in response to acute volume expansion indicated that a similar amount of IR-NT-ANP was released in SCH 39370-treated rats as in control rats for each given increase in right atrial pressure. These findings show that NEP inhibitors increase plasma ANP concentration by dose dependently inhibiting ANP degradation, but they did not affect the release of the peptide from the heart.

c. ANTIHYPERTENSIVE ACTIVITY OF NEUTRAL ENDOPEPTIDASE INHIBITORS. In view of the ability of NEP inhibitors to potentiate responses to ANP and enhance plasma ANP levels, their effects on hemodynamic variables and renal function have been studied in several experimental models. The DOCA/salt hypertensive rat has been used in many studies because plasma ANP levels are elevated and the renin-ANG system is suppressed, two conditions which optimize the expression of ANP-like effects by a drug that inhibits ANP metabolism. Subcutaneous or oral administration of NEP inhibitors SCH 39370, SCH 34826, and SCH 42495 significantly reduced blood pressure by 25 to 50 mm Hg in the DOCA/salt hypertensive rat (Sybertz et al., 1989, 1990a,b, 1991; Vemulapalli et al., 1991). The antihypertensive effect was sustained for at least 4 h. The acute antihypertensive response to NEP inhibitor SCH 34826 was associated with a significant reduction in cardiac output and no change in peripheral vascular resistance or heart rate (Sybertz et al., 1990a). Similarly, NEP inhibitor 28603 decreased blood pressure in DOCA/salt hypertensive rats with a significant reduction of tissue NEP activity and an increase in plasma IR-ANP concentration and urinary sodium and cGMP excretion (Seymour et al., 1991b,c). The antihypertensive effect of NEP inhibitors in the DOCA/salt hypertensive rat has been confirmed by others with different NEP inhibitors (Haslanger et al., 1989; Sybertz et al., 1989, 1990b; Seymour et al., 1990b; Shepperson et al., 1991). Interestingly, preliminary experiments also suggest that chronic SCH 34826 and SCH 42495 treatments for 1 month may reduce left ventricular hypertrophy in SHR without affecting blood pressure (Monopoli et al., 1991).

In the SHR, NEP inhibitors SCH 39370 and SCH 34826 were devoid of acute antihypertensive activity (Sybertz et al., 1989, 1990a), but SCH 34826 elicited a modest, but significant, reduction of blood pressure when administered twice daily over a 5-day period (Sybertz et al., 1990a). Furthermore, other NEP inhibitors (SQ 29072) have been shown to decrease mean arterial pressure in SHR (Seymour et al., 1990a, 1991d), although responses were smaller than in the DOCA/salt rats. SCH 39370 alone also decreased blood pressure in normal sodium-replete sheep associated with increased plasma IR-ANP and cGMP levels and reduced plasma aldosterone and cortisol levels (Charles et al., 1991c). In contrast, inhibition of NEP activity by thiorphan (Koepke et al., 1989; Olins et al., 1989; Trapani et al., 1989; Webb et al. 1989), SQ 28.603 (Seymour et al., 1991b) and SCH 39370 (Sybertz et al., 1989; Kukkonen et al., 1992) had no antihypertensive effect in normotensive animals. Thus, hypertensive rats appear to be more sensitive to

depressor effects of NEP inhibitors. Furthermore, activation of the biological processes responsible for the blood pressure response appear to require a latency period, because maximal hypotensive activity has been observed 2 to 4 h after drug administration (Sybertz et al., 1990a). Thus, a longer administration of NEP inhibitors to chronically elevate plasma ANP concentration may be needed to cause alterations in hemodynamic variables in normal rats, as has been documented for exogenous ANP infusions in both experimental animals and humans (Brenner et al., 1990; Hollister and Inagami, 1991; de Zeeuw et al., 1992). Yet, thiorphan infused for 10 days in conscious SHR had no effect on mean arterial blood pressure, and thiorphan did not affect hypotensive responses to $ANP_{103-126}$ (Koepke et al., 1990a).

In a recent study, Jin et al. (1992) examined the effect of chronic administration of SCH 34826 in NaCl-sensitive SHR. SCH 34826 (90 mg/kg/day by cavage for 4 weeks) prevented the increase in arterial pressure in response to 8% NaCl in NaCl-sensitive SHR but had no effect on blood pressure in 1% NaCl-fed NaCl-sensitive SHR; plasma IR-ANP concentrations increased by 63 and 68% in the 1 and 8% NaCl groups, respectively, in response to SCH 34826 (Jin et al., 1992). Acute administration of SCH 34826 also reduced the NaCl-induced increase in blood pressure in 8% NaCl-fed NaCl-sensitive SHR but had no effect in NaCl-sensitive SHR fed the 1% NaCl diet for 3 weeks. Taken together, the results show that NEP inhibitors, especially acutely, elicit ANP-like hemodynamic effects in volume-expanded models of hypertension, such as in the DOCA/salt hypertensive rat and NaCl-sensitive SHR.

d. RENAL EFFECTS OF NEUTRAL ENDOPEPTIDASE INHIBITORS. Ura et al. (1987) have shown that infusion of phosphoramidon causes an increase in urinary Na^+ excretion rate, an effect attributed to inhibition of breakdown of kinins or ANP. Lafferty et al. (1989) found that phosphoramidon infusion in rats with reduced renal mass resulted in a marked diuresis and natriuresis as well as an increase in GFR and filtration fraction, effects similar to those seen after infusion of ANP. These renal effects were associated with augmentation of plasma IR-ANP levels and urine cGMP excretion (Lafferty et al., 1989). Others have also reported similar results of NEP inhibitors on renal function in normal animals. Thiorphan increased urinary sodium excretion in anesthetized and conscious normal rats (Koepke et al., 1989; Trapani et al. 1989; Smits et al., 1990a), and acetorphan was diuretic and natriuretic in SHR and Wistar rats under basal conditions and during volume load (Bralet et al., 1990). In anesthetized normal dogs, SQ 28603 produced increases in urine flow and sodium excretion with marked increases in urinary excretion of cGMP and IR-ANP, consistent with decreased renal excretion and increased biological activity of ANP (Margulies et al., 1990). In conscious mice and anesthetized rats, UK 69578 and UK 73967 were shown to potentiate the natriuresis and diuresis induced by saline infusion (Danilewicz et al., 1989; Shepperson et al., 1991). In Dahl-S rats, candoxatril-induced natriuresis was associated with a marked increase in the urinary IR-ANP excretion (Suzuki et al., 1992). Finally, NEP inhibition by SQ 28603 produced natriuresis, diuresis, and a significant increase in cGMP excretion without affecting mean arterial pressure and plasma IR-ANP levels in normal monkeys (Seymour et al., 1991a).

The results of the effects of NEP inhibition in experimental animals with CHF suggest that they are more effective than infusion of exogenous ANP. In anesthetized dogs with experimental heart failure induced by pacing, SQ 28603 resulted in a diuresis and natriuresis with increases in the fractional excretion of sodium and in urinary IR-ANP and cGMP concentrations; these changes occurred in the absence of increases in GFR, renal blood flow, or mean arterial pressure (Cavero et al., 1990). Accordingly, in a chronic CHF model produced by coronary artery ligation in the rat, SCH 39370 treatment resulted in an increase in urine volume and urinary IR-ANP and cGMP concentrations (Tikkanen et al., 1990). In this model, plasma IR-ANP and cGMP concentrations increased to the initial level even after 6 days of treatment with SCH 39370 (Helin et al., 1991). Similar results were reported by Trippodo et al. (1991), who found that SQ 28603 caused a significant natriuresis/diuresis and decrease in central venous pressure in rats with moderate to large myocardial infarcts (but not in normal rats), whereas mean arterial pressure, cardiac output, and GFR remained unchanged. Furthermore, a rapid 6-fold increase in urinary sodium excretion accompanied by a pronounced increase in urinary cGMP was observed in the rat aortovenocaval fistula model of cardiac failure (Wilkins et al., 1990a). Thiorphan produced a 3-fold increase in urinary sodium excretion in the cardiomyopathic hamsters but did not in normal animals (Smits et al., 1990b). In an ovine heart failure model, bolus injection of SCH 39370 caused natriuresis and diuresis and decreased left atrial pressure but had little effect on other hemodynamic parameters (Fitzpatrick et al., 1992). Results of these studies support an important therapeutic role for NEP inhibitors in persons with CHF, especially because blood pressure remained unchanged in the above-mentioned studies and the response was consistently greater in animals with heart failure than control animals.

Several studies have suggested that NEP inhibitors may modulate renal function in hypertensive rats. NEP inhibitor SCH 34826 elicited a significant diuresis during a 3-h period in the DOCA/salt hypertensive rat (Sybertz et al., 1990a). Urine sodium excretion tended to be elevated; however, this was not significant. In another study of DOCA/salt hypertensive rats, SCH 34826 had significant natriuretic effects and tended to increase urinary

sodium excretion (Vemulapalli et al., 1991). These renal effects are noteworthy, given the fact that blood pressure was significantly reduced by SCH 34826. SCH 39370 and SCH 34826 also significantly enhanced the renal excretion of IR-ANP and cGMP (Sybertz et al., 1989, 1990a,b, 1991; Vemulapalli et al., 1991). SQ 29072 elicited a modest natriuretic response in the hydrated SHR and a prolonged increase in urinary excretion of sodium, cGMP, and volume in the DOCA/salt rats (Seymour et al., 1990b). The early increase in urine volume and sodium excretion that preceded the depressor response to SQ 28603 may have attenuated the subsequent reduction of mean arterial pressure (Seymour et al., 1991b). Thiorphan produced a greater increase in urinary sodium, cGMP, and ANP concentrations in SHR than WKY rats without affecting blood pressure in either strain (Hirata et al., 1991). Moreover, NEP inhibitors also potentiated the natriuretic effects of pathophysiological concentrations of ANP in dogs (Margulies et al., 1990).

Within the kidney, endopeptidase EC 24.11 is present in the brush border membranes in high concentrations. Under normal conditions, filtered ANP is degraded by endopeptidase within the renal brush border membrane. Inhibition of this enzyme would result in passage of ANP to the distal portions of the nephron where it can exert its action. NEP inhibition in animals with CHF probably has renal tubular actions because it produced natriuresis and diuresis independently of GFR and renal blood flow and the increase in plasma IR-ANP concentration; furthermore, exogenous ANP failed to enhance sodium and water excretion (Cavero et al., 1990). The magnitude of the plasma IR-ANP concentration increase in response to thiorphan in rats with atrioventricular fistulas could not be explained by an increase in plasma levels of ANP (Wilkins et al. 1990a). Extrarenal inhibition of endopeptidase provides an alternative explanation for potentiation of the renal effects of ANP, because the enzyme has widespread distribution throughout the body. In support of this, the delaying effect of NEP inhibitors on plasma ANP clearance was augmented in nephrectomized rats, showing that the effect of NEP inhibitors on ANP disposition may occur at extrarenal sites (Sybertz et al., 1989, 1991; Chiu et al., 1991; Shepperson et al., 1991). Thus, both renal and extrarenal site inhibition of ANP breakdown may contribute to the renal effects of NEP inhibitors.

e. ROLE OF ATRIAL NATRIURETIC PEPTIDE IN RESPONSE TO NEUTRAL ENDOPEPTIDASE INHIBITORS. The role of ANP in the antihypertensive response to NEP inhibitors in the DOCA/salt hypertensive rat has been assessed by measuring plasma and urine IR-ANP and cGMP concentrations. SCH 34826 significantly increased plasma IR-ANP levels (753 versus 451 pg/ml in the control group) 1 h after administration (Sybertz et al., 1990a). However, plasma IR-ANP levels were not significantly different from those in the vehicle-treated control group of animals when measurements were made 3 h after administration, a time at which the antihypertensive action of SCH 34826 was fully manifested. Thus, there was a dissociation in plasma ANP levels from the antihypertensive effect of SCH 34826. Furthermore, plasma IR-ANP concentrations following i.v. injection of ANP were higher than after SCH 32615 injection (Sybertz et al., 1990b, 1991), although they produced equivalent hypotensive responses. Results of other studies also suggest that the hypotensive or renal effects of NEP inhibitors are not related to an increase in plasma IR-ANP levels in experimental animals (Smits et al., 1990a).

Evidence for a causal role of ANP in the antihypertensive action of NEP inhibitors comes from studies in which ANP antibodies were used. A polyclonal antiserum to ANP injected i.v. abruptly reversed the antihypertensive effect of SCH 34826 or SCH 42495 in the DOCA/salt hypertensive rat (Sybertz et al., 1990b, 1991). The natriuresis associated with parenteral administration of candoxatrilat is largely abolished by pretreatment with ANP antisera (Shepperson et al., 1991), and similarly, coadministration of antiserum with candoxatrilat abolished the antihypertensive response to the endopeptidase inhibitor in the conscious DOCA/salt hypertensive rat (Shepperson et al., 1991). The antiserum raised against ANP prevented the diuretic and natriuretic effects of thiorphan after volume loading in anesthetized rats (Bralet et al., 1990). Pretreatment with monoclonal antibody against ANP inhibited the increase in urinary sodium and cGMP excretion induced by thiorphan (Wilkins et al., 1990a; Bralet et al., 1991), providing further evidence that ANP is a major factor in initiating the natriuretic response to the drug. In addition, pretreatment of rats with rabbit anti-rat ANP antiserum appears to inhibit changes in hematocrit, plasma protein concentration, and blood pressure in response to SQ 28603 (Valentin et al., 1992). The major tissues contributing to the actions of NEP inhibitors on blood pressure remain to be determined, but the kidney, blood vessel wall, and adrenal gland are likely targets. Interestingly, NEP is present in vascular tissues particularly in the endothelium (Soleilhac et al., 1992) and thus ideally localized to regulate circulating ANP levels.

As described before, NEP metabolizes numerous vasoactive peptides (Erdös and Skidgel, 1989). NEP also cleaves BNP (Vogt-Schoden et al., 1989). Conceivably, enhancement of the levels of one or more of these potential substrates may have produced some of the effects of NEP inhibitors. However, neither bradykinin nor enkephalins participate in the antihypertensive response to NEP inhibitors because specific antagonists of bradykinin [(thi^{5,8}-D-phe^{7})bradykinin] and opiates (naltrexone) failed to alter the blood pressure response to NEP inhibition by SCH 39370, SCH 34826, and SCH 42495 (Sybertz et al., 1989, 1990b, 1991). Furthermore, SCH

34826, SQ 29072 and SQ 28603 did not affect the depressor responses to bradykinin or the pressor effects of ANG I, ANG II, ET-1, or AVP (Seymour et al., 1986; Sybertz et al., 1990a; Gardiner et al., 1992). However, although bradykinin is not involved in the antihypertensive response, there is evidence to suggest a role for bradykinin in the renal response to NEP inhibition (Ura et al., 1987; Smits et al., 1990a; Sybertz et al., 1990b, 1991; Bralet et al., 1991). Endopeptidase accounted for about 53 to 74% of the degradation of intrarenal kinins, and the inhibition of endopeptidase by phosphoramidon increased renal kinin excretion (Ura et al., 1987). Furthermore, the potentiation of renal actions of ANP by thiorphan or phosphoramidon was abolished by a specific antagonist of the bradykinin receptor (Smits et al., 1990a; Bralet et al., 1991; Sybertz et al., 1991). In contrast, Hirata et al. (1991) reported that thiorphan-induced natriuresis was attenuated by ANP antiserum but not by a bradykinin receptor antagonist. Furthermore, the bradykinin antagonist alone reduced urine sodium and volume excretion and attenuated the renal responses to ANP in anesthetized normotensive rats (Sybertz et al., 1991), showing that the effect of the antagonist is not specific for NEP inhibitors. Thus, to what extent peptides other than ANP participate in the pharmacological activities of NEP inhibitors requires further examination.

f. ATRIAL NATRIURETIC PEPTIDE-LIKE ACTIONS OF NEUTRAL ENDOPEPTIDASE INHIBITORS IN HUMANS. There are presently few data documenting the effects of EC 24.11 inhibition in humans. However, the results are consistent with the evidence from animal experiments showing that NEP inhibition potentiates ANP bioactivity. In a study by Richards et al. (1991a), the effects of chronic administration of candoxatril, an orally active inhibitor of NEP, were assessed in normal volunteers on the metabolic clearance and the biological effects of a near-physiological dose of exogenous ANP given by infusion. Two groups of eight men received a constant infusion of human ANP_{99-126} (2.5 pmol/kg min for 2 h) on the 5th day of taking placebo or candoxatril (25 mg every 12 h or 100 mg every 12 h) in randomized, double-blind, placebo-controlled, crossover study. The studies were completed without adverse effects. Inhibition of NEP with subnatriuretic and modestly natriuretic doses of candoxatril significantly reduced the metabolic clearance of exogenous ANP and enhanced the biological actions of ANP, as evidenced by the following: (*a*) augmentation of plasma and urinary cGMP, (*b*) enhanced natriuresis and the renal filtration fraction, and (*c*) borderline but significant tachycardia and hypotension (Richards et al., 1991a). Urinary ANP immunoreactivity was also clearly enhanced. The changes in metabolic clearance were substantial (reductions of 41 and 59%) and suggest that the endopeptidase system plays an important role in the clearance of ANP from plasma. All effects were more pronounced at the higher dose of candoxatril (Richards et al., 1991a). Thus, in normal humans, chronic inhibition of NEP with candoxatril significantly reduced the metabolic clearance rate of exogenous ANP and enhanced biological responses to exogenous ANP, as demonstrated earlier in experimental animals.

The profile of biological effects of NEP inhibitors in normal volunteers closely parallels those previously reported with low-dose infusions of ANP in humans and experimental animals. Northridge et al. (1989) reported the effects of parenteral administration of UK 69578, a potent and specific NEP inhibitor, after a single dose and after an 8- to 12-h follow-up period in both normal volunteers and patients with heart failure in a double-blind study. All doses were well tolerated, and no adverse effects were reported. In normal volunteers, UK 69578 (2 mg/kg) doubled plasma ANP concentrations between 1 and 2 h after i.v. infusion of the compound, and the higher doses prolonged this effect for 4 to 8 h (Northridge et al., 1989). Plasma renin concentrations reduced and sodium excretion increased in a dose-dependent manner and correlated with the peak increase in plasma IR-ANP concentration. A similar increase in plasma IR-ANP concentration with an increase in urine volume and urinary sodium excretion after i.v. administration of UK 69578 to normal volunteers was reported by Jardine et al. (1990). The maximal increase in plasma IR-ANP levels was seen after the dose of 0.5 mg/kg with little effect from further increases in doses. The elimination $t_{1/2}$ of the UK 69578 was 1.1 h (Jardine et al., 1990).

Other compounds have also been reported to increase plasma IR-ANP concentration with diuresis and natriuresis in normal humans. Gros and colleagues (1989) administered two doses of acetorphan to normal volunteers in a randomized, double-blind, placebo-controlled study. Peak elevation of plasma IR-ANP concentration (double baseline) occurred 2 h after oral administration, coinciding with peak plasma enzyme inhibition. The increase was still significant 4 h after 100 mg of acetorphan and 8 h after 300 mg of acetorphan administration. The increase in IR-ANP levels was accompanied by dose-related increases in urine volume and sodium excretion (Gros et al., 1989). Single oral doses of SCH 34826 (400 to 1600 mg) significantly increased urinary excretion of sodium, phosphate, and calcium in normotensive volunteers with high sodium intake without affecting the renal excretion of potassium and systemic blood pressure (Burnier et al., 1991). The renal response to SCH 34826 occurred without any concurrent increase in plasma IR-ANP concentration but with a significant elevation of urinary cGMP and IR-ANP concentrations (Burnier et al., 1991).

The effects of an orally active inhibitor, UK 79300 (25 or 50 mg orally for 24 h), were investigated in six healthy volunteers in a random order, single-blind study (Richards et al., 1990). UK 79300 enhanced plasma IR-ANP

concentrations in association with suppression of both plasma renin activity and aldosterone concentrations, a clear natriuresis, and an increase in cGMP excretion. The observed mean enhancement of plasma IR-ANP concentration was little more than 3 to 5 pmol/liter even at the peak effect of UK 79300 (Richards et al., 1990). Blood pressure and heart rate were not affected by UK 79300, which agrees with the results of studies of sustained low-dose infusion of ANP in which significant decreases in blood pressure did not occur in normotensive men (Richards et al., 1988). However, a hypotensive effect was observed in hypertensive subjects (Janssen et al., 1989; Richards et al., 1989a; Cusson et al., 1990). These studies suggest that NEP inhibition elicits specific renal effects of ANP in normal individuals, resulting in a natriuretic response.

In a recent study, Richards et al. (1991b) examined the effects of prolonged (4 days) inhibition of endopeptidase by candoxatril on renal, endocrine, and cardiovascular function in normal humans. Inhibition of endopeptidase by continued administration of UK 79300 led to a sustained enhancement of urine and plasma cGMP concentration. In contrast, plasma IR-ANP concentration was hardly significantly enhanced by low doses of UK 79300, and the weak trend toward enhanced levels observed with the higher dose of the inhibitor was not significant (Richards et al., 1991b). Natriuresis was observed at the higher dose and was transient, the heart rate increased, systolic blood pressure tended to decrease, and the renin-ANG-aldosterone system was neither suppressed nor activated. The basal renin, ANG II, and aldosterone values were low, however, probably explaining why further suppression did not occur (Richards et al., 1991a).

The minimal effect on plasma IR-ANP levels observed after 4 and 5 days of administration of candoxatril (Richards et al., 1991a,b) is in contrast to the obvious increase on day 1 of administration with this compound at similar doses and under similar conditions (Richards et al., 1990), showing that plasma ANP levels return toward baseline values during sustained administration of NEP inhibitors. As discussed before, this may reflect activation of clearance receptor function and other possible elimination pathways or a reduction in endogenous secretion of ANP. The observation that prolonged NEP inhibition reduced metabolic clearance (Richards et al., 1991b) suggests that endogenous ANP secretion is reduced with continued enzyme inhibition. This may be mediated by contraction of the circulating volume, with consequent decreases in atrial pressure, i.e., the initial increase in plasma IR-ANP concentration may exert feedback via hemodynamic mechanisms to reduce ANP secretion. However, this hypothesis requires confirmation with measurements of left and right atrial pressures, intravascular volume, and NH_2-terminal peptide concentration during chronic administration of NEP inhibitors. The fact that cGMP levels were sustained suggests that NEP inhibitors prolong the cellular effect of ANP, thus producing a greater action for a given circulating concentration of ANP.

In patients with heart failure, NEP inhibitors have been reported to increase circulating ANP concentrations with an associated diuresis and natriuresis. Parenteral administration of UK 69578 (10 to 400 mg) resulted in a 2- to 5-fold increase in plasma IR-ANP concentrations 1 and 2 h after administration with an associated increase in natriuresis and diuresis as well as reductions in mean right atrial pressure and pulmonary wedge pressures in humans with mild left ventricular dysfunction (Northridge et al., 1989, 1990). There was no change in heart rate, mean arterial pressure, cardiac output, or echocardiographic dimensions after UK 69578 administration. Plasma levels of IR-ANP also significantly increased by 126% after 200 mg and by 141% after 400 mg of UK 79300 in patients with CHF, but these changes were not accompanied by significant effects on left ventricular hydraulic load (Kromer et al., 1991). An orally active endopeptidase inhibitor, sinorphan (30 or 60 mg given three times a day), elicited a 2- to 3-fold increase in plasma IR-ANP levels which peaked at 3 h in 12 patients with severe heart failure (Kahn et al., 1990). The increase in plasma IR-ANP concentration was associated with a lowered pulmonary capillary wedge pressure, whereas cardiac output, heart rate, and mean arterial pressure remained unchanged. Thus, NEP inhibition appears to be an effective means of increasing ANP levels acutely in patients with heart failure and may improve hemodynamic effects, which are similar to those produced by exogenous ANP administration in patients with CHF. Even though plasma ANP concentration is already increased in patients with CHF, ANP infusion has beneficial effects [reduced cardiac filling pressure, diuresis, and natriuresis (Molina et al., 1988)], although blunted renal effects in those with chronic heart failure have been demonstrated (for recent reviews, see Perrella et al., 1991a; de Zeeuw et al., 1992). Interestingly, the orally active prodrug of UK 69578, candoxatril, also increases plasma IR-BNP concentration concomitantly with an elevation of plasma IR-ANP concentration in patients with CHF (Lang et al., 1991b, 1992b), raising the possibility that BNP may contribute to some of the biological effects of NEP inhibitors.

Because plasma ANP levels are elevated in many patients with essential hypertension and a constant infusion of exogenous ANP in low doses reduces blood pressure in hypertensive patients, the efficacy of NEP inhibitors has been tested in patients with mild to moderate essential hypertension. Singer et al. (1991) studied the renal and hormonal effects of candoxatril in eight patients with untreated essential hypertension receiving high sodium (350 mmol/day) and low sodium (10 mmol/day) diets. The basal plasma IR-ANP and IR-NT-ANP

levels were higher in patients receiving the high compared with the low sodium diet. NEP inhibition increased plasma IR-ANP concentration in those receiving both diets within 1 h and remained elevated (2-fold) for up to 6 h, whereas NT-ANP levels remained unchanged; the increase in plasma IR-ANP levels in absolute values was greater in patients receiving the high than the low sodium diet (Singer et al., 1991). In addition, the increase in sodium excretion with candoxatril in patients receiving the high sodium diet was greater, both as a percentage and in absolute amounts, compared with that in patients receiving the low sodium diet. Plasma renin activity was suppressed by treatment in the low sodium diet group but not the high sodium diet group, and blood pressure did not change in the 6 h after a single oral dose of candoxatril (Singer et al., 1991). Thus, sodium intake appears to be a major determinant of the natriuretic and plasma ANP response to NEP inhibition in hypertensive patients.

The effects of three doses of candoxatril (10, 50, and 200 mg) was examined in a prospective, double-blind, placebo-controlled trial in untreated essential hypertensive patients (O'Connell et al., 1992). Plasma IR-ANP concentrations increased significantly in all patients within 2 h of candoxatril administration compared with placebo, although peak and integrated ANP levels were similar at all three doses. A significant natriuresis was seen after 200 mg candoxatril, whereas urinary potassium excretion, blood pressure, and heart rate remained unchanged at all doses of the drug studied (O'Connell et al., 1992). In contrast, administration of SCH 34826 (400 mg, four times a day) significantly reduced supine systolic and diastolic blood pressure by 16 and 12 mm Hg, respectively, in 12 black hypertensive individuals (Sybertz, 1991). The antihypertensive effect was sustained throughout the 14 days of treatment. Heart rate was not altered. Interestingly, urinary excretion of sodium and volume were not altered, suggesting that the individuals could maintain normal excretory function in spite of large reductions of blood pressure (Sybertz, 1991). In a small open study of 12 hypertensive patients, the antihypertensive effect of sinorphan was maintained for more than 1 month (Lefrancois et al., 1990). The blood pressure results support those in animal studies and imply an antihypertensive activity of NEP inhibition in humans.

In conclusion, there is considerable interest in the possible therapeutic potential of inhibitors of NEP in patients with CHF and hypertension in view of the elevated plasma ANP levels and the biological effects of ANP. Yet, many significant questions remain unanswered. The results suggest that changes induced by NEP inhibition on ANP metabolism and activity at the target tissue level (kidney, vasculature) are more important than increments in plasma ANP concentrations. In acute studies in humans and experimental animals, the biological effects of NEP inhibitors closely parallel those reported during sustained low-dose infusion of exogenous ANP (tables 5 and 6). NEP inhibitors are probably effective in states of elevated levels of endogenous ANP, such as CHF and hypertension. The precise role of ANP, other natriuretic peptides, or other vasoactive peptide substrates of NEP on the actions of the inhibitors in animals and humans as well as the renal and extrarenal sites of action of NEP inhibitors require further clarification. Differences in the pharmacological properties of the compounds have not been assessed to date. Finally, although the full therapeutic potential for this new class of agents in the treatment of cardiovascular and renal diseases remains to be defined, NEP inhibition represents a novel means of cardiorenal modulation and may yield a new class of therapeutic agents for treatment of cardiovascular diseases such as CHF and hypertension.

3. Effect of combined inhibition of ANP_C receptors and neutral endopeptidase. Combined inhibition of NEP and ANP_C receptors markedly alters ANP metabolism. C-ANF_{4-23} and SCH 39370, an NEP inhibitor, in combination produced the greatest effect on the pharmacokinetics of ^{125}I-ANP (Chiu et al., 1991); the clearance was reduced to a minimum (from 61 to 4 ml/min/100 g body weight), and the plasma level of ^{125}I-ANP was considerably augmented with prolongation of its $t_{1/2}$ from 1.4 to 10.4 min, showing that concomitant occupancy of the ANP_C receptors and inhibition of NEP have a synergistic effect on the pharmacokinetics of ANP. Inhibition of NEP may also protect C-ANF_{4-23} from degradation and thereby enhance its biological effects, as suggested by the observation that the NEP inhibitor SQ 28603 prevented C-ANF_{4-23} cleavage by NEP in vitro (Seymour et al., 1991b). Plasma IR-ANP increased 2-fold in conscious normal rats (Koepke et al., 1989) and SHR (Koepke et al., 1990b) during coadministration of SC-46542 and thiorphan, whereas administration of ANP_C receptor ligand or NEP inhibitor alone had no effect on plasma IR-ANP concentration (Koepke et al., 1989). Furthermore, in conscious DOCA/salt hypertensive rats, only the combination of C-ANF_{4-23} and SQ 28603 significantly increased plasma IR-ANP concentrations; yet, the combination of C-ANF_{4-23} and SQ 28603 was ineffective in increasing plasma IR-ANP levels in conscious normotensive rats (Seymour et al., 1991b). These results suggest that part of the basis for the modest effect of NEP inhibitors on plasma ANP levels is the efficiency of the C-type receptor mechanism in clearing ANP from the plasma after NEP has inhibited its degradation and vice versa. The data also emphasize the dynamic interplay between ANP_C receptors and NEP in the physiological disposition of ANP.

Recent studies have shown that coadministration of C-type receptor ligands and NEP inhibitors produce a greater diuresis, natriuresis, and depressor response than administration of either agent alone, probably reflecting

TABLE 5

*Effect of some NEP inhibitors on plasma levels of ANP and other hormones in human**

Study	Drug	Dose	Administration	n	ANP	Plasma renin activity	cGMP	Aldosterone
Normal volunteers								
Gros et al., 1989	Acetorphan	100 or 300 mg	p.o., single	8	↑			
Northridge et al., 1989	UK 69578	0.025–10 mg/kg	i.v., single	8	↑	↓		
Jardine et al., 1990	UK 69578	0.025–10 mg/kg	i.v., single	16	↑	↓		→
Richards et al., 1990	Candoxatril	25 or 50 mg × 2	p.o., single	6	↑	↓		↓
Burnier et al., 1991	SCH 34826	400–1600 mg	p.o., single	8	→		↑	
Richards et al., 1991b	Candoxatril	25 or 100 mg × 2	p.o., 4 days	8	↑→	↑↓	↑	↑
Patients with heart failure								
Northridge et al., 1989	UK 69578	10–400 mg/kg	i.v., single	6	↑	→		
Northridge et al., 1990	UK 69578	10–400 mg/kg	i.v., single	6	↑	→		
Kahn et al., 1990	Sinorphan	30–60 mg × 3	p.o., single	12	↑	↓		
Kromer et al., 1991	UK 79300	200–400 mg × 1	p.o., single	14	↑			
Lang et al., 1991b	Candoxatril	10–200 mg	p.o., single	7	↑			
Lang et al., 1992b	Candoxatril	10–200 mg	p.o., single	7	↑	→		↓
Hypertensive patients								
Lefrancois et al., 1990	Sinorphan	25–200 mg × 2	p.o., 42 days	12				
Singe et al., 1991	Candoxatril	200 mg × 1	p.o., single	8	↑	↓		↑
O'Connell et al., 1992	Candoxatril	10–200 mg × 1	p.o., single	12	↑	→		→

* Abbreviations and symbols: p.o., oral; single, 1 day; ↑, increase; ↓, decrease; →, no change.

TABLE 6

*Effect of some neutral endopeptidase inhibitors on renal function and hemodynamics in human**

Study	Drug	Blood pressure	Heart rate	PAWP	RAP	U_{Na}	U_V	U_{cGMP}
Normal volunteers								
Gros et al., 1989	Acetorphan					↑	↑	
Northridge et al., 1989	UK 69578					↑		
Jardine et al., 1990	UK 69578					↑	↑	
Richards et al., 1990	Candoxatril	→	→			↑	→	↑
Burnier et al., 1991	SCH 34826	→	→			↑	→	↑
Richards et al., 1991b	Candoxatril	→	↑			↑	→	↑
Patients with heart failure								
Northridge et al., 1989	UK 69578	→	→	↓	↓	↑	↑	
Northridge et al., 1990	UK 69578	→	→	↓	→	↑	↑	
Kahn et al., 1990	Sinorphan	→	→	↓				
Kromer et al., 1991	UK 79300	→						
Lang et al., 1991b	Candoxatril							
Lang et al., 1992b	Candoxatril	→						
Hypertensive patients								
Lefrancois et al., 1990	Sinorphan	↓	→					
Singer et al., 1991	Candoxatril	→	→			↑	↑	
O'Connell et al., 1992	Candoxatrl	→	→			↑	↑	

* Abbreviations and symbols: PAWP, pulmonary artery wedge pressure; RAP, right atrial pressure; U_{Na}, urinary sodium excretion; U_V, urine volume; U_{cGMP}, urine cGMP; ↑, increase; ↓, decrease; →, no change. When measured (Northridge et al., 1989; 1990; Kahn et al., 1990), cardiac output did not change.

greater accumulation of endogenous ANP in target tissues due to the concomitant inhibition of both receptor-mediated clearance and NEP (Koepke et al., 1989, 1990b; Seymour et al., 1991b; Vemulapalli et al., 1991; Kukkonen et al., 1992). The coadministration of SC-46542 and thiorphan increased the depressor, natriuretic, and diuretic sensitivities to low-dose $ANP_{103-126}$ (Koepke et al., 1989). The same combination also produced a marked increase in urinary sodium and cGMP excretion, whereas mean arterial pressure, GFR, and plasma cGMP concentration remained unchanged in SHR and WKY rats; the change in urine sodium excretion was greater in SHR than WKY rats, and each drug alone had no effect on these variables (Koepke et al., 1990b). The combination of C-ANF_{4-23} and SCH 34826 (Vemulapalli et al., 1991) or C-ANF_{4-23} and SQ 28603 (Seymour et al., 1991b) augmented the following: (*a*) hypotension; (*b*) increases in plasma cGMP concentration; and (*c*) urinary excretion of cGMP, sodium, and water. In support of these studies, the combination of the NEP inhibitor SCH 39370 and the ANP_C receptor ligand C-ANF_{4-23} significantly decreased blood pressure and right atrial pressure in conscious normotensive rats, even though neither drug individually affected either variable (Kukkonen et al., 1992). Furthermore, the combination produced an additive increase in basal plasma IR-ANP concentration, in spite of the fact that the combined treatment decreased blood pressure and right atrial pressure, although mod-

estly, which may decrease the stimulus for endogenous ANP secretion from the heart. Thus, these data suggest a positive interaction between the two pathways by which ANP is thought to be inactivated in vivo. The results of these experiments also show that relatively modest increases in plasma ANP concentration are biologically effective, which further supports the significance of this hormone as a modulator of blood pressure and plasma volume.

Yet, although acute coadministration of ANP_C receptor ligands and NEP inhibitors results in greater effects than administering either alone, the combination may not be an effective long-term treatment for hypertension. Long-term coinfusion of thiorphan and SC-46542 (but not single infusion) resulted in decreased arterial pressure in SHR (Koepke et al., 1990a). However, this antihypertensive response was not sustained; coinfusion of SC-46542 and thiorphan decreased mean arterial pressure for a few days, after which it returned toward control levels but never attained pretreatment values during 10 days of infusion (Koepke et al., 1990a). Of note, when $ANP_{103-126}$ (ANP_{99-126} was not studied) was infused together with SC-46542 and thiorphan, the decrease in mean arterial pressure was more transient and smaller than that found during coinfusion of SC-46542 and thiorphan.

One possible explanation for the lack of therapeutic efficacy in long-term treatment may be that ANP is inactivated by other elimination pathways, which were activated in the presence of combined C-type receptor ligand and NEP inhibitor infusion (fig. 10). In agreement with this, when SCH 39370 and $C\text{-}ANF_{4-23}$ were administered together, plasma IR-ANP concentration increased less in response to volume expansion in the combination group than when each substance was administered individually (Kukkonen et al., 1992). Furthermore, Johnson et al. (1989) reported the presence of peptidases in vascular smooth muscle and endothelial cells that were not inhibited by SCH 39370, which cleave the COOH-terminal tripeptide from ANP. Thus, whether the inhibition of the main elimination pathways of ANP may be overridden by other metabolic pathways, which are not detected or have less physiological importance because of more rapid attack by ANP_C receptors and endopeptidase under basal conditions, remains to be investigated. Furthermore, endogenous levels of ANP may be maintained at steady state by a negative feedback control mechanism, i.e., the amount of ANP released from the heart could be decreased at high plasma ANP levels to restore equilibrium. Supporting this hypothesis, Kukkonen et al. (1992) found that the plasma NT-ANP concentration in response to volume load tended to increase less in the presence of the combination of SCH 39370 and $C\text{-}ANF_{4-23}$ than in the vehicle group. Jin et al. (1990) reported that $C\text{-}ANF_{4-23}$ produced a 3-fold increase in plasma IR-ANP concentration in normal rats but only a 2-fold increase in hypoxia-adapted rats, a situation with elevated endogenous ANP levels. However, more work, particularly in vitro experiments, are needed to demonstrate the possible role of a negative feedback mechanism on the maintenance of steady-state plasma ANP concentrations during the administration of inhibitors of ANP elimination.

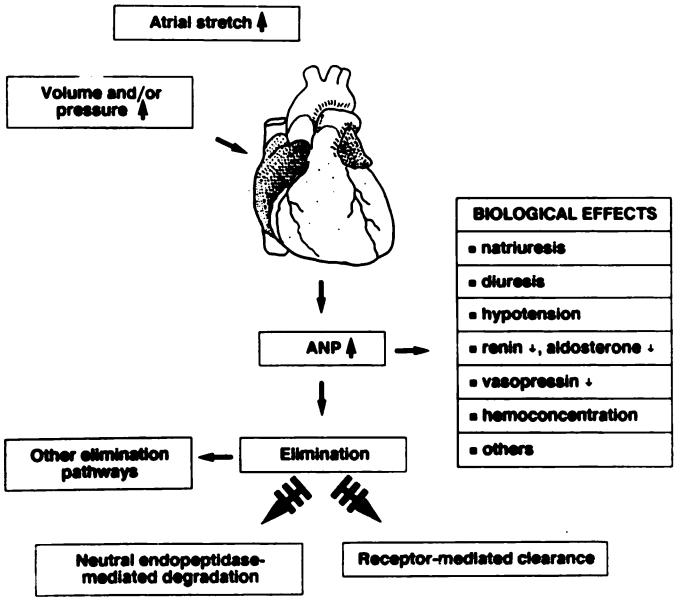

FIG. 10. Mode of action of inhibitors of ANP elimination.

In summary, given in combination, ANP_C receptor ligands and NEP inhibitors produce a greater elevation of plasma ANP as well as more pronounced acute hemodynamic and renal effects than either compound given alone (fig. 10). Thus, a likely explanation for the modest effect of NEP inhibitors on plasma ANP is the fact that activity of the ANP_C receptor buffers any changes in plasma ANP induced by NEP inhibition and vice versa. Clearly, although the rate of release of ANP from cardiac myocytes is a major regulator of plasma ANP concentration, enzymatic degradation and receptor-mediated removal of ANP from the circulation in several tissues, including the kidney, lung, and vascular smooth muscle, may also affect circulating plasma ANP concentrations. The preliminary results also suggest that the combined inhibition of NEP and clearance receptors may yet result in an increase in the activity of possible alternate elimination pathways. The higher levels of bioactive ANP found in all cases whether drugs were added alone or in combination demonstrate that preventing the inactivation may be of therapeutic value in situations with elevated endogenous plasma ANP levels.

VI. Pharmacological Implications

A. Rationale for the Pharmacological Use of Atrial Natriuretic Peptide

Since the initial identification of ANP in 1981, there has been an explosion of information concerning the molecular biology, biochemistry, physiology, and phar-

macology of this hormone. The genes encoding ANP and ANP-like peptides from many diverse species have been cloned and characterized. The biosynthetic steps required for processing the precursor molecule into a mature hormone have been ascertained, and the structural features of the resultant peptide that are critical for function have been defined. The predominant as well as other appropriate stimuli for ANP synthesis and release have been identified. The guanylyl cyclase family of proteins has been shown to serve as receptors for ANP, subsequently generating increased amounts of the second messenger cGMP. The importance of receptor-mediated clearance and enzymatic degradation by NEP for elimination of ANP from the circulation has been defined. The studies in which the production, storage, release, and elimination of ANP in physiological and pathophysiological conditions were examined have been particularly important in understanding the role of this endogenous peptide and in evaluating the possible usefulness of ANP as a therapeutic agent.

The conclusion that ANPs or drugs mimicking the action of endogenous ANP could be valuable therapeutic agents is based on the following findings: (*a*) cardiac biosynthesis, storage, and release of ANP are altered in a variety of pathophysiological states; (*b*) failure of ANP secretion to further increase in response to cardiac overload in some clinical conditions; (*c*) the diversity of pharmacological effects of ANP (blood pressure, renal actions, renin secretion, aldosterone secretion, vasopressin release); and (*d*) positive effects of monoclonal antibodies or ANP antiserum in some disease states. The continuing interest in the role of ANP in the pathophysiology and treatment is reflected by the fact that most of the nearly 3000 articles published between 1989 and 1991 were related to measurements of plasma ANP levels in different pathophysiological situations.

Because ANP is secreted in response to myocyte stretch, it is not surprising that the role of ANP has been explored in disorders of volume and pressure regulation including CHF, hypertension, renal failure, and liver diseases (table 7). Tikkanen et al. (1985a) were the first to demonstrate that ANP is increased in plasma in patients with CHF and that there was a correlation between severity of the condition and circulating levels of ANP. Others have confirmed and extended these observations by showing elevated plasma IR-ANP levels in patients with CHF. There is a positive correlation between plasma IR-ANP values and the severity of the disease, New York Heart Association functional class, right atrial pressure, and pulmonary capillary wedge pressures and an inverse correlation with cardiac index and stroke volume (Tikkanen et al. 1985a; Bates et al., 1986; Burnett et al. 1986; Cody et al. 1986; Dietz et al., 1986; Katoh et al. 1986; Lang et al. 1986; Raine et al. 1986; Riegger et al. 1986; Rodeheffer et al., 1986; Schiffrin and Taillefer, 1986; Saito et al. 1987c; Yasue et al., 1989). Plasma IR-ANP levels also show good correlation with the prognosis of heart failure (Gottlieb et al., 1989). In the elderly, elevated IR-ANP levels may be a marker for significant systolic and/or diastolic dysfunction, with consequent risk for CHF (Davis et al., 1992). Effective therapy for CHF leads to reductions in plasma IR-ANP levels that usually are proportional to the improvement in clinical status and cardiac performance (Katoh et al. 1986; for a recent review, see Perrella et al., 1991a). As discussed earlier, the increase in plasma IR-ANP levels and the activation of cardiac ANP gene expression represent compensatory responses, decreasing cardiac workload by hemodynamic and renal effects. This interpretation has stimulated investigators to evaluate the therapeutic value of further increases in plasma ANP levels in patients with heart failure.

The observations that patients with CHF have volume overload, increased preload, and increased systemic vascular resistance, despite often markedly elevated plasma IR-ANP levels, implies an insensitivity to the effects of

TABLE 7
Pathophysiological conditions that may be associated with changes in plasma levels of ANP

Increase in plasma ANP concentration
Any condition resulting in volume or pressure overload is a candidate
Cardiac diseases
CHF
Cardiomyopathies
Myocarditis
Valve diseases: rheumatic heart disease
Coronary heart disease: myocardial ischemia and infarction
Arrhythmias
Paroxysmal atrial tachycardia
Atrial fibrillation and flutter
Congenital heart defects
Restrictive disorders
Pericarditis
Cardiac tamponade
Drug-induced cardiac diseases
Hypertension
Essential hypertension
Secondary, pulmonary, portal hypertension
Kidneys diseases
Chronic renal failure
Nephrosis
Cirrhosis
Primary aldosteronism
Cushing's disease
Preeclampsia, toxemia of pregnancy, premenstrual syndrome
Hyperthyroidism
Syndrome of inappropriate antidiuretic hormone secretion
Bartter's syndrome
Diabetes mellitus
Burn injury
Subarachnoid hemorrhage
Decrease in plasma ANP concentration
Any condition resulting in a decrease of intravascular blood volume or cardiac filling pressure is a candidate
Hypothyroidism
Gordon's syndrome
Atrial standstill

endogenous ANP and that further elevation of these levels by exogenous ANP would be ineffective. Indeed, CHF is characterized by markedly blunted diuretic and natriuretic responses to exogenous ANP despite increases in GFR (Cody et al., 1986). However, despite the high plasma IR-ANP levels, administration of exogenous ANP has occasionally been found to lead to improved hemodynamic, renal, and hormonal responses (Cody et al. 1986; Saito et al. 1987a,c; Molina et al., 1988; Giles et al., 1991), probably by reducing preload and afterload.

The fact that sodium and water retention occur in patients with heart failure despite high circulating levels of ANP implies that the ANP elevation is inadequate for the degree of heart failure and suggests that relative deficiency of ANP contributes to the development of CHF. Studies of severe CHF in experimental animal models showed a lack or attenuation of the ANP response to volume and/or pressure overload (Mendez et al., 1987; Ruskoaho and Leppäluoto, 1988a; Redfield et al., 1989b; Moe et al., 1990, 1991). Failure of acute cardiac overload in animal experiments to elevate plasma IR-ANP levels was not supported by studies in patients with CHF (Uretsky et al., 1990). However, recent experiments by Volpe et al. (1991c) showed that, in patients with DCM and mild cardiac dysfunction, plasma ANP levels do not increase in response to sustained isotonic volume load. Importantly, when circulating levels of ANP were maintained elevated by exogenous infusion, the natriuretic, renin-aldosterone inhibitory and vascular effects caused by saline loading in the heart failure group were significantly augmented as compared to those with lower levels of ANP. These findings suggest that ANP deficiency may be a cause of the altered renal, hormonal, and vascular responses in CHF.

Findings from antibody studies support a therapeutic role for ANP. Infusion of specific anti-ANP antibody to rats with CHF secondary to myocardial infarction acutely suppressed renal salt and water excretion (Awazu et al. 1989; Drexler et al., 1990), suggesting that fluid retention would have been even more marked in the absence of chronically elevated ANP levels. A modulatory role of high levels of circulating ANP in heart failure is also suggested by experiments in autoimmune rats (Greenwald et al., 1988) and in animals treated with NEP inhibitors. Thus, a role has been advanced for ANP in the long-term treatment of CHF based on these observations.

The profile of ANP activity suggests that it could be used in the treatment of essential hypertension and other hypertensive diseases. In clinical hypertension, plasma ANP levels vary widely (for reviews, see Espiner and Richards, 1989; Hollister and Inagami, 1991; Sagnella et al., 1991; Weidmann et al., 1991; de Zeeuw et al., 1992). There is considerable evidence showing that a substantial proportion of patients with essential hypertension have elevated circulating ANP levels, suggesting important compensatory mechanisms. The pathogenesis of essential hypertension may also involve a deficiency in ANP secretion (Ferrari et al., 1990; Volpe et al., 1991a). Studies by Ferrari et al. (1990) have suggested that, compared with the healthy offspring of normotensive parents, offspring of hypertensive parents have plasma IR-ANP levels that are unaltered by low salt intake and often fail to increase normally in response to a high salt intake. Moreover, in borderline to moderate, uncomplicated essential hypertension, plasma ANP levels are often low relative to existing blood pressure. Accordingly, experimental models of hypertension, such as SHR, have normal plasma ANP levels in young animals despite elevated blood pressure and high levels in the advanced hypertensive phase complicated by ventricular hypertrophy (Ruskoaho and Leppäluoto, 1988a). A deficient response of circulating ANP concentration to high sodium intake or acute volume expansion has been also noted in SHR (Jin et al., 1988a; Ruskoaho and Leppäluoto, 1988a).

The pathophysiological significance of endogenous ANP in hypertension has been examined by repetitive administration of monoclonal antibody raised against ANP in experimental models of hypertension (Itoh et al., 1989b, 1991; Yang et al., 1990). Weekly i.v. administration of antibodies significantly augmented the increase in blood pressure in SHR and aggravated hypertension in DOCA/salt rats. Chronic blockade of endogenous ANP with weekly administration of monoclonal ANP antibody also aggravated development of pulmonary hypertension and right ventricular hypertrophy (Raffestin et al., 1992). These results suggest a compensatory role of increased secretion for ANP in these hypertensive models and a therapeutic role for ANP in hypertension. ANP administration in hypertensive patients has usually resulted in blood pressure reduction (for recent reviews, see Hollister and Inagami, 1991; de Zeeuw et al., 1992). Bolus or short-term ANP infusions reduce blood pressure in human subjects with essential or secondary hypertension. Longer term infusions of ANP (from several hours to 7 days at doses that often achieved plasma ANP concentrations in the upper physiological range) exert beneficial hypotensive or renal effects in normal volunteers and in patients with essential hypertension (Espiner and Richards, 1989; Hollister and Inagami, 1991; Sagnella et al., 1991). In addition, i.v. ANP might be useful in the treatment of severe hypertension in hospitalized patients. However, in acute studies, ANP has been shown to have a narrow therapeutic index, with lower doses having little effect and slightly higher doses inducing intolerable hypotension (Weidmann, 1989; de Zeeuw, 1992).

Chronic regulation of the cardiovascular system by ANP has also been investigated by generating transgenic mice with elevated ANP levels in the systemic circulation. A line of mice was produced by Steinhelper et al. (1990a) that carries a chimeric transgene linking the 5′-

regulatory sequences of the mouse transthyretin promoter, a strong, constitutively active promoter in liver tissue, to mouse ANP-coding sequences. Their results suggest that persistently high levels (at least 8-fold higher than normal) of ANP reduce arterial blood pressure by 25 to 30 mm Hg without inducing diuresis, natriuresis, or changes in heart rate in transgenic mice (Steinhelper et al., 1990a; Field et al., 1991). Yet, after acute blood volume expansion, the diuretic and natriuretic responses were markedly enhanced in the transgenic animals compared with their nontransgenic siblings (Field et al., 1991). These are important findings given the conflicting data reported from studies of long-term ANP infusion in normal and hypertensive animals. The relative ANP "deficiency," acute and short-term antihypertensive effects of sustained ANP infusions and NEP inhibitors, and experiments with monoclonal antibodies and transgenic animals represent a rational basis for the treatment of some forms of hypertension with agents that activate the ANP system.

Given its ability to augment GFR, an attractive prospect for ANP is in the reversal of acute renal failure. In various models of acute renal failure in the rat, including postischemic, norepinephrine-, cisplatin-, and uranyl-nitrate models (Cole et al., 1985; Schafferhans et al., 1986; Capasso et al. 1987; Nakamoto et al., 1987; Shaw et al., 1987; Cogan, 1990), ANP infusion increases GFR. Windus et al. (1989) demonstrated the ability of ANP infusion to augment sodium excretion by 65% in a cohort of patients with renal failure. Infusion of specific anti-ANP antibody into rats with reduced renal mass led to marked reductions in fractional and absolute excretion of sodium under conditions in which GFR and renal plasma flow were unchanged (Ortola et al., 1987). Short-term infusion of exogenous ANP into cirrhotic subjects with ascites and edema resulted in modest transient natriuresis and diuresis which, however, was often complicated by troublesome hypotension. The latter side effect seems to be less prominent when ANP is given as a bolus dose (Weidmann et al., 1989; Cody, 1990). Generally, the profile suggests that ANP may prove useful in the treatment of many other conditions in which normal pressure-volume relationships are disturbed (table 7). ANP has also been reported to relax tracheal rings in vitro, and it may thus have value in the treatment of bronchoconstrictor states, such as asthma and the respiratory distress syndromes (for further references, see Brenner et al., 1990; de Zeeuw et al., 1992).

Is there any clinical state in which excessive ANP activity could be harmful and should be blocked? Glomerular hyperfiltration has been suggested to be a factor linked to the development of diabetic glomerulopathy, and increased ANP levels have been linked to the glomerular hyperfiltration in experimental animals (Ortola et al., 1987). Administration of antiserum to ANP decreased the GFR in diabetic rats, suggesting that an ANP antagonist might be used to decrease the progression of renal disease in diabetic patients (Ortola et al., 1987).

B. Therapeutic Possibilities to Affect Atrial Natriuretic Peptide Hormonal System

The high potency of ANP and its complete metabolism into its constituent amino acid subunits suggests that it is an ideal "natural" therapeutic agent. However, peptides and peptide hormones, like ANP, are themselves poor drugs. An ideal drug should be active when administered orally, should be readily absorbed from the intestine into the circulation, and should rapidly penetrate into the target organs. Thus, the susceptibility of ANP to enzymatic attack in the gut and the circulation with a resulting short $t_{1/2}$ and the need to administer ANP i.v. are the principal arguments against its use as a drug. In the rat, ANP or its analogs may be administered either intramuscularly or subcutaneously (Tosti-Croce et al., 1989), whereas in humans, Crozier et al. (1987b) did not observe any diuretic or natriuretic effect after subcutaneous administration of ANP, despite noting a 3-fold elevation of plasma IR-ANP levels. Nevertheless, the short-term parenteral use of ANP will be limited to hospitalized patients. Several alternatives could be used, such as nasal spray, ointments, or rectal suppositories; indeed, Shionoiri and Kaneko (1986) and Delabays et al. (1989) found that intranasally administered ANP both lowered blood pressure and induced diuresis. The availability of such preparations will make outpatient short-term administration of ANP or its analogs possible; however, they will probably not replace oral agents in the long-term treatment of CHF or hypertension.

Because the use of ANP as a therapeutic agent is limited by the fact that it must be given parenterally, it is obvious that the development of drugs that mimic or block its effects should be important as scientific probes as well as therapeutic agents. Developing effective drugs to influence peptide systems, however, is extremely difficult, and thus the finding of potent, orally effective drugs influencing ANP would be a significant breakthrough. The theoretical possibilities for mimicking or blocking the action of ANP are summarized in table 8. At present, it is not possible to selectively affect synthesis or secretion of ANP. Nevertheless, knowledge of the

TABLE 8
Mechanisms to influence and mimic biological actions of ANP

Drugs affecting the synthesis of ANP
Drugs affecting the processing and release of ANP
Drugs affecting ANP_A receptors
ANP_A receptor agonists
ANP_A receptor antagonists
Drugs preventing elimination of ANP
ANP_C receptor ligands: C-ANP_{4-23}, SC-46542, etc.
NEP inhibitors: candoxatrilat, SCH 34826, SQ 29072, etc.
Inhibitors of other elimination pathways
Drugs enhancing actions of cGMP
cGMP-phosphodiesterase inhibitors

properties and functions of synthesis, processing enzymes, and release might enable development of effective drugs for diverse diseases associated with alterations in the ANP hormonal system. Theoretically, if a specific enzyme responsible for processing ANP exists, drugs might be specifically developed that affect only the biosynthesis of proANP allowing control of the hormone production in the heart.

1. ANP_A receptor analogs. It has been possible to develop forms of ANP with altered structure, truncated and conformationally restricted analogs of ANP, that retain some or several of the pharmacological properties of the parent peptide and have an enhanced circulatory $t_{1/2}$ (Schiller et al., 1987; Bovy et al., 1989, 1990; Goghari et al., 1990; Pollock and Opgenorth, 1990; Holleman et al., 1991). These new agents may facilitate the development of therapeutic agents that interact with the ANP receptors. Holleman recently reported the design and synthesis of reduced-size analogs of ANP, Arg^6-Cha^8 ANP_{6-15} Phe-Arg-Cys-NH_2 (A-68828), which potently stimulates the synthesis of cGMP and possess full agonist activity. A-68828 in vivo is only slightly less natriuretic (1:20 to 1:50) than ANP_{99-126} and inhibits adrenocorticotropic hormone-induced aldosterone release to a greater extent than ANP_{99-126} but has only mild hypotensive activity (Holleman et al., 1991). This relative lack of a hypotensive effect may be important if the renal effects would be expressed in the absence of hypotension, e.g., in the treatment of acute renal failure or CHF. Assessment of the efficacy of these and other analogs in clinical states such as acute renal failure in humans is obviously needed. Although a number of analogs are available, there is still need for more specific substances that are more resistant to biodegradation than the natural peptides. Theoretical modeling of bioactive conformations and synthesis of stabilized molecular forms of peptides play an important role in the search for new drugs. Nevertheless, it is difficult to synthesize a peptide that is readily absorbed from the gastrointestinal tract.

Recently, enormous advances have been made in our understanding of signal transduction through activation of guanylyl cyclase. Yet, the more complete understanding of the particular physiological roles of the various natriuretic peptides and their receptors and their tissue-specific localization will be important from the therapeutic standpoint. In conjunction with the development of receptor-specific agonists, a thorough understanding of receptor distribution may eventually allow targeting of such agonists to particular tissues for clinical purposes, avoiding the unwanted effects of ANP.

2. Inhibitors of atrial natriuretic peptide metabolism. Because sustained responses to ANP are at present only obtained by i.v. infusions of the peptide, a method of increasing endogenous levels of the hormone, such as inhibition of a crucial inactivation pathway in vivo, appears to provide the most promising alternative for regulating ANP levels. The structure of the ANP_C receptor is known, and it will be a major challenge to develop orally active analogs that are potent and specific ANP_C receptor ligands. An increasingly important task is the search for inhibitors of enzymatic degradation of ANP. The aim of these efforts is to find molecules that can be administered orally that specifically inhibit the degradation of endogenous ANP. However, current data suggest that, when such a pathway is eliminated, others compensate. The simultaneous inhibition of receptor-mediated pathways, endopeptidase, and other metabolic pathways would produce a greater pharmacological effect than interference with each individual system. Results of animal experiments suggest that in CHF and in hypertension mediated primarily by volume overload, endopeptidase inhibitors are most effective. Human studies are currently in progress testing the orally active inhibitors as possible agents for treatment of chronic cardiovascular diseases including CHF and hypertension.

The use of elimination inhibitors together with low doses of ANP analogs or in combination with other agents currently used in the treatment of cardiac overload remains to be explored. As discussed before, inhibition of one ANP elimination pathway may be overridden by other metabolic pathways, e.g., during long-term NEP inhibition. Thus, the use of a combination of NEP inhibitors and ACE or kallikrein inhibitors may be useful in the treatment of hypertensive diseases and CHF. The increased activity of the renin-ANG system is thought to play a major role in the diminished renal response to ANP, presumably in the pathogenesis of salt retention and edema formation in heart failure. Accordingly, ACE inhibitor treatment restored the hemodynamic responsiveness to ANP in the coronary ligation rat model of heart failure (Raya et al., 1989; Lee et al., 1992), the natriuretic response to exogenous administration of ANP in sodium-retaining rats with an atrioventricular fistula model of heart failure (Abassi et al., 1990), the renal hemodynamic and excretory responses to NEP inhibition in dogs with experimental CHF produced by rapid ventricular pacing (Margulies et al., 1991), and the renal responsiveness to ANP in dogs with compensated high-output heart failure (Villarreal et al., 1992). The enhancement of ANP action by ACE inhibition has been reported also in humans (Agabiti-Rosei et al., 1987). This interaction between ANP and the renin-ANG system merits further clinical studies. It is possible that the combination of ACE inhibition and ANP could be used as a therapeutic strategy to treat patients with heart failure and hypertension. Mixed inhibitors of ACE and NEP have already been designed (Gros et al., 1991; Seymour et al., 1991d). Two of them, glycopril and alatriopril, displayed a combination of biological activities in vivo by preventing the hypertensive action of ANG I in rats and by increasing urinary excretion of water,

sodium, and cGMP in rats subjected to extracellular volume expansion (Gros et al., 1991).

An alternative possibility to enhance the activity of endogenous ANP is to inhibit degradation of cGMP (for a recent review, see Wilkins and Needleman, 1992). M+B 22948, a selective cGMP phosphodiesterase inhibitor, greatly potentiates the natriuretic response to coadministered ANP and enhances the natriuretic activity of endogenous ANP during acute volume loading (Wilkins et al., 1990b). In addition, M+B 22948 was more effective in stimulating the natriuresis in the atrioventricular fistula rat with a minimal decrease in blood pressure compared with the sham-operated animals (Wilkins et al., 1990a). cGMP-specific phosphodiesterase inhibitors also potentiated the diuretic and natriuretic effects of exogenous ANP (Weishaar et al., 1990). These studies suggest that combined inhibition of cGMP degradation and NEP activity would be more effective than administration of either alone in the long-term treatment of cardiovascular diseases. Furthermore, the greater efficacy of NEP inhibitors and inhibition of cGMP degradation in the presence of elevated circulating ANP suggests that, when diuresis progresses and circulating ANP levels decrease, the renal effects of these compounds also attenuate, protecting the patient from possible overdiuresis and the concomitant side effects.

3. Atrial natriuretic peptide antagonists or inhibitors. These agents may be useful in the characterization of the role of ANP in physiological and pathophysiological states. At present, there are no antagonists of the actions of ANP safe for use in humans. However, animal studies have shown that treatment with a monoclonal antibody directed against ANP blunts the natriuretic response to a sodium load (Hirth et al., 1986). Heparin, which is known to bind atrial peptides and interfere with their biological activities (Wei et al. 1987a), decreased the magnitude of natriuresis and diuresis observed after relief of bilateral urinary tract obstruction (Purkerson et al. 1989), suggesting a role for ANP in the treatment of salt and volume retention in urinary tract obstruction. Kambayashi et al. (1989a) found that an analog of β-ANP blocked the stimulation of smooth muscle guanylate cyclase by ANP and was devoid of biochemical or physiological agonist activity. The importance of these and other ANP antagonists (Abell et al., 1989b; Kitajima et al., 1989; von Geldern et al., 1990; Weber et al., 1991; Wyss et al., 1991; Morishita et al., 1992; Sano et al., 1992a,b) remains to be clarified.

In summary, whether or not ANP is involved in the pathophysiology of CHF, hypertension, or other pressure and volume overload states, its potency as a pharmacological agent in altering cardiovascular and renal function makes it an exciting candidate for treating patients with excessive cardiac preload and afterload. Unfortunately, being a peptide, it must be given i.v.. So far, the overall benefit from short-term, parenteral administration of ANP in a number of diseases has been variable and has often been limited by the development of hypotension. The NEP inhibitors, clearance receptor ligands, and ANP antagonists provide important additional insights into the physiological and pharmacological actions of ANP. Further studies with these agents may yield new therapeutic approaches to the treatment of common cardiovascular diseases such as CHF and hypertension.

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