

# Autocrine-Paracrine Endothelin System in the Physiology and Pathology of Steroid-Secreting Tissues

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## I. Introduction

Endothelins (ETs)<sup>2</sup> are a family of endogenous peptides, mainly secreted by endothelial cells, which exert a potent vasoconstrictor and pressor activity, acting through two classes of receptors named ETA and ETB. ETs were originally isolated from porcine aortic endothelium (Yanagisawa et al., 1988; Inoue et al., 1989), but subsequent studies revealed that ETs are synthesized and their receptors are present in a variety of tissues, where they play important physiological and pathophysiological roles. At present, ETs are thought to regulate the cardiovascular apparatus and blood pressure, kidney excretion, pulmonary function, endocrine gland secretion, and the development of tissues derived from embryonic neural crest lineage and of the central and peripheral nervous systems. The ability of all of these tissues to synthesize ETs and the fact that blood concentrations of these peptides, not exceeding the picomolar range under both physiological and pathophysiological conditions (Kohno et al., 1990a; Weitzberg et al., 1991; Letizia et al., 1996; Maeda et al., 1997; Herold et al., 1998; Kobayashi et al., 1998; Makino and Kamata, 1998), are well below those able to evoke appreciable biological effects, led to the contention that ETs almost exclusively act through autocrine/paracrine mechanisms.

ETs are known to be involved in the functional regulation of neuroendocrine axes, among which are the hypothalamo-pituitary-adrenal and the hypothalamo-pituitary-gonadal axes, acting on either their central or peripheral branch. Evidence has been accumulated that indicates that the ET genes and their receptors are expressed in both adrenal glands and endocrine components of the gonads, the function of which they variously modulate. Because adrenocortical cells, Leydig intersti-

<sup>2</sup> Abbreviations: ET, endothelin; ETA, endothelin receptor A; ACTH, adrenocorticotrophic hormone; ANG-II, angiotensin-II; ANP, atrial natriuretic peptide; APA, aldosterone-producing adenoma; AVP, arginine-vasopressin; BP, systolic blood pressure;  $[Ca^{2+}]_i$ , intracellular (cytosolic) calcium concentration; CO, cyclooxygenase; DAG, diacylglycerol; db, dibutyl; DHEA, dehydroepiandrosterone; ECE, endothelin-converting enzyme; ETB, endothelin receptor B; FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; HSD, hydroxysteroid dehydrogenase; IL, interleukin;  $IP_3$ , inositol-triphosphate; IVF-ET, in vitro fertilization-embryo transplantation; LH, luteinizing hormone; LI, like immunoreactivity; L-NAME, N<sup>G</sup>-nitro-L-arginine; LO, lipoxygenase; MAPK, mitogen-activated protein kinase;  $[Na^+]_i$ , intracellular (cytosolic) sodium concentration; NO, nitric oxide; NOS, nitric oxide synthase; OLC, ouabain-like compound; PAC, plasma aldosterone concentration; PBC, plasma corticosterone concentration; PG, prostaglandin; PK, protein kinase; PL, phospholipase; PMA, phorbol 12-myristate 13-acetate; pp, prepro; PRA, plasma renin activity; P450arom, aromatase; P450c11, 11 $\beta$ -hydroxylase; P450c17, 17 $\alpha$ -hydroxylase; P450c18, aldosterone synthase; P450c21, 21-hydroxylase; P450scc, cholesterol side-chain cleaving enzymes; RT-PCR, reverse transcription polymerase-chain reaction; SER, smooth endoplasmic reticulum; STX, sarafotoxin; TK, tyrosine kinase; ZF, zona fasciculata; ZF/R, zona fasciculata-reticularis; ZG, zona glomerulosa; ZR, zona reticularis.

tial cells of the testis, and ovarian granulosa/lutein cells possess common morphological and functional characteristics, they are frequently called steroid-secreting cells. Steroid-secreting cells produce cholesterol-derived hormones through complex multistep pathways involving enzymes located in both mitochondria and smooth endoplasmic reticula (SER) and release newly formed steroid hormones constitutively as soon as they are produced (i.e., without the possibility of any cytoplasmatic storage). Despite these similarities, steroid-secreting cells display marked differences in their response to ETs.

Since their discovery in the late 1980s, ETs and their receptors have been the topic of several excellent review articles, emphasizing their possible role in health and disease (LeMonnier de Gouville et al., 1989; Simonson and Dunn, 1990, 1991; Sakurai et al., 1992; Battistini et al., 1993; Haynes and Webb, 1993; Haynes et al., 1993; Lotersztajn, 1993; Bax and Saxena, 1994; Rubanyi and Polokoff, 1994; Goto et al., 1996; Levin, 1996a; Ohlstein et al., 1996; Schiffrin, 1996; Dashwood et al., 1997; Webb, 1997; Webb and Meek, 1997; Stjernquist, 1998; Webb et al., 1998). Because of the very potent vasoconstrictor effects of ETs, a large part of these surveys dealt with the cardiovascular system. The possible involvement of ETs in the functional regulation of the endocrine system also has been surveyed, although far less extensively (MacRae and Bloom, 1992; Kennedy et al., 1993; Masaki, 1993; Takuwa, 1993; Naruse et al., 1994a; Inagami et al., 1995; Stojilkovic and Catt, 1996; Ehrhart-Bornstein et al., 1998). However, only a few surveys specifically dealt with the autocrine/paracrine role played by ETs in the functional regulation of steroid-secreting tissues. Of the latter, two concerned the adrenal cortex (Nussdorfer et al., 1997a; Remy-Jouet et al., 1998), and two others concerned the endocrine gonads, although marginally (Saez, 1994; Stojilkovic, 1996).

After a brief summary of the current knowledge of the molecular and cell biology of ETs and steroid-secreting tissues, in the following sections of this survey we will describe and discuss findings indicating that locally synthesized ETs are involved in the autocrine/paracrine control of the secretion and growth of steroid-secreting tissues. Then we shall review the possible mechanisms underlying these actions of ETs, as well as those regulating the local release of ETs. Finally, evidence for the possible involvement of ETs in the pathophysiology of steroid-secreting tissues will be surveyed.

## II. Biology of Endothelins and Steroid-Secreting Cells

### A. Endothelins and their Receptors

1. *Endothelins*. ETs are peptides formed by 21 amino acids, with four cysteine residues forming two intramolecular disulfide bonds. The ET family includes three distinct isoforms, ET-1, ET-2, and ET-3. Their amino

acid sequences are very similar, but their tissue distribution differs considerably. Interestingly, soon after the first publication on ETs, the structure of peptides isolated from the venom of the Israeli burrowing asp *Atractaspis engaddensis*, termed sarafotoxins (STXs), was shown to have remarkable similarities to ETs (Kloog and Sokolovsky, 1989). The four STXs known so far (STXa, STXb, STXc, and STXd) have 21-amino acid residues and possess considerable sequence homology to ETs in their carboxyl terminus, which is crucial for binding to their specific receptors (Fig. 1). Although all ETs of the family are synthesized as prepro(pp) proteins of about 200 amino acids, which share a high degree of sequence homology, and a two-step processing pathway, they are encoded by three distinct genes.

a. **ENTOTHELIN GENES AND THEIR REGULATION.** Three genes encoding ET-like sequences were identified in the mammalian genome and shown to encode the precursors of ETs, ppET-1, ppET-2, and ppET-3 (Inoue et al., 1989). Subsequent studies mapped the three genes in the human genome on chromosomes 6, 1, and 20, respectively (Bloch et al., 1989a,b,1991; Hoehe et al., 1993). Detailed molecular investigation of the ppET gene promoter regions demonstrated sequences necessary for high levels of transcription (Lee et al., 1990, 1991), as well as for the specificity of tissue expression (Benatti et al., 1994). It was also established that several extracellular factors could influence ET production both positively and negatively. Factors activating the phospholipase (PL) C/protein kinase (PK) C pathway may enhance ET-1 production, through binding of the proto-oncogenes *jun* and *fos*

to the activator protein-1 (AP-1) transcription regulatory element of the ppET-1 promoter. These factors include insulin, thrombin, oxidized low density lipoprotein, angiotensin-II (ANG-II), vasopressin, and ET-1 itself (Kohno et al., 1990b, 1992; Emori et al., 1991, 1992; Lee et al., 1991; Boulanger et al., 1992; Benatti et al., 1994). Factors, possibly acting through the cGMP-mediated pathway and thought to inhibit ET-1 production, include atrial natriuretic peptide (ANP), parathyroid hormone-related protein, and lipopolysaccharide (Fujisaki et al., 1995; Jiang et al., 1996; Kaddoura et al., 1996; Wada et al., 1996). Shear stress ambivalently affects ppET-1 gene transcription, with low stress (<2 dyne/cm<sup>2</sup>) and high stress (>6 dyne/cm<sup>2</sup>) enhancing and blunting it, respectively (Malek and Izumo, 1992; Kuchan and Frangos, 1993; Malek et al., 1993, 1997; 1994; Davies, 1995). Because inhibitors of either nitric oxide (NO) synthesis or guanylate cyclase abolished this latter effect, it was suggested that high shear stress releases NO, which in turn would inhibit ET-1 production via cGMP generation (Rubanyi and Polokoff, 1994). Because Ca<sup>2+</sup> chelators also blunt ET-1 release from endothelial cells (Emori et al., 1992), it is likely that cGMP-induced inhibition of ET-1 is due to its ability to impair intracellular Ca<sup>2+</sup> availability.

b. **BIOSYNTHESIS.** All biologically active isopeptides of the family are generated via a two-step processing (Fig. 2), which first involves sequential cleavage by signal peptide peptidases and furin-like convertases to form the physiologically inactive ET precursors, the big-ETs (for references, see Rubanyi and Polokoff, 1994). The big-ETs are polypeptides of 37 to 41 amino acids, which show species-specific differences in their C-terminal 22–41 sequence. They are cleaved exactly at the Trp<sup>21</sup>-Val/Ile<sup>22</sup> peptide bond to form physiologically active ETs by a specific endopeptidase referred to as endothelin-converting enzyme (ECE). After its first cloning from bovine tissues, the cDNA species coding for two ECE

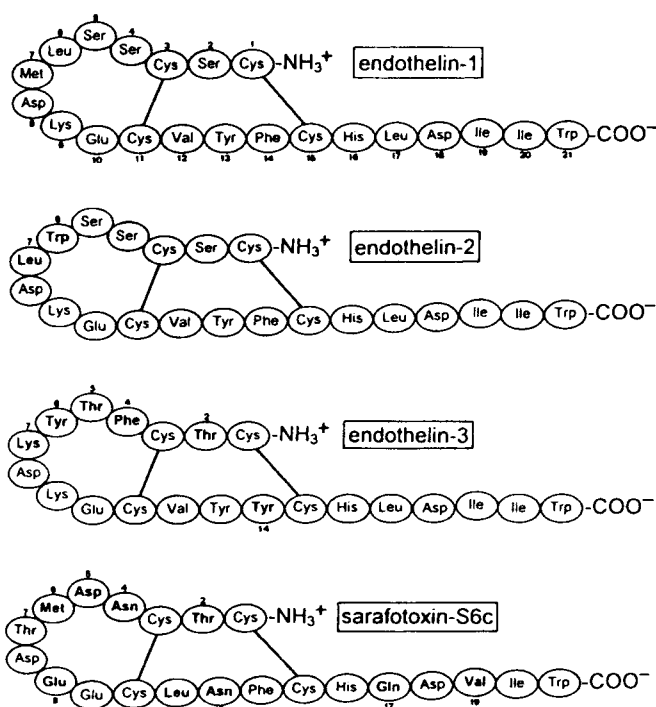


FIG. 1. Amino acid sequence of human ETs and STXc. Amino acid substitutions with respect to ET-1 are indicated.

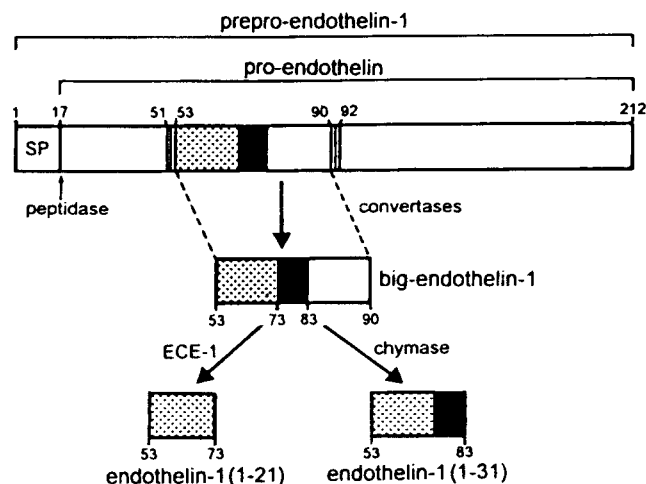


FIG. 2. Biosynthesis and processing of ET-1 from ppET-1. SP, signal peptide.

forms, termed ECE-1 and ECE-2, have been isolated (for review, see Schweizer et al., 1997). Both ECEs belong to a new family of zinc metalloendopeptidases, which are inhibited by phosphoramidon, and display remarkable structural similarities with a short N-terminal domain, a single transmembrane segment, and a large C-terminal part, including the active site of the enzyme. Very recently, the purification of a novel ECE specific for big-ET-3 from bovine iris was reported (Hasegawa et al., 1998). The genomic organization of the human ECE-1 gene, which is localized on chromosome 1p36 (Valdenaire et al., 1995) and more precisely on 1p36.2.3 (Albertin et al., 1996), has recently been unraveled. Shear-stress response elements have been identified on the regulatory region of the human ECE-1 gene, which provides a rational basis for the coupling of shear stress with ET-1 synthesis (Valdenaire et al., 1995). Moreover, an ETB-mediated suppressive effect of ET-1 on ECE-1 mRNA expression has been demonstrated in cultured rat pulmonary endothelial cells, thereby suggesting the existence of a negative feedback mechanism regulating ET-1 synthesis (Naomi et al., 1998). Although the ET biosynthetic pathway involving ECEs is the predominant one and the most widely investigated, there is the possibility that big ETs are also specifically cleaved by chymase at the Tyr<sup>31</sup>-Gly<sup>32</sup> bond (Nakano et al., 1997; Fig. 2). This cleavage would lead to novel ET(1-31) isopeptides, which were shown in vitro to evoke a less potent but slower and longer-lasting contraction of porcine coronary artery and rat aorta than ETs(1-21). Interestingly, at variance with ET-1, the vasoconstrictor effect of ETs(1-31) was inhibited by either the ETA receptor antagonist BQ-485 or the ETB receptor antagonist BQ-788, which suggests that these novel peptides might act in a different manner from classic ETs, possibly through different receptors (Kishi et al., 1998).

c. CLEARANCE. The plasma half-life of ET-1 in mammals is less than 1.5 min because it is efficiently cleared by splanchnic, renal, and pulmonary vascular beds (Hemsen et al., 1995; Kobayashi et al., 1998). On binding to its receptors on cell membrane, ET-1 undergoes internalization followed by rapid degradation, through still incompletely understood enzymatic pathways (Perez-Vizcaino et al., 1995). Because the plasma levels of ET-1 were increased by both mixed ETA/ETB- and ETB-specific, but not ETA-specific receptor antagonists, it has been proposed that the ETB receptor may play a role as a clearance receptor (Fukuroda et al., 1994; Teerlink et al., 1994; Gandhi et al., 1998; Kobayashi et al., 1998). This role of ETB receptors needs to be taken into account in the interpretation of results of experiments using ETB-specific antagonists, where a potentiation of ETA-mediated effects is likely to supervene.

2. *Endothelin Receptors.* Within 2 years of the initial report of ET discovery, the genes encoding two different specific receptors termed ETA and ETB, were identified and cloned (Arai et al., 1990; Sakurai et al., 1990; Lin et

al., 1991). ET-1 is deemed to exert its effects via the activation of these main receptor subtypes, although molecular variants, most likely deriving from alternative splicing, have been reported (Elshourbagy et al., 1996; Miyamoto et al., 1996).

a. BIOCHEMISTRY AND MOLECULAR BIOLOGY. The ETA and ETB receptors at first were differentiated pharmacologically based on their affinity for the isopeptides of the ET family. The ETA receptor is characterized by its very high (subnanomolar) affinity for ET-1 and ET-2 and a 70- to 100-fold lower affinity for ET-3. The ETB receptor is non-isopeptide-selective, as it binds with high and similar affinity to all isopeptides of the ET family. As mentioned above, ETA and ETB receptors are coded by two different genes, which are structured into eight and seven exons and have been mapped on chromosomes 4 and 13, respectively. Because these genes show considerable similarities in their structural organization (Elshourbagy et al., 1996), they are thought to derive from a common ancestral precursor gene. Both receptors belong to the superfamily of the G protein-coupled receptors and entail seven transmembrane domains, an extracellular N terminus, and an intracytoplasmic C terminus. The two receptors differ not only in the N terminus, but also in the C terminus, which is crucial for their coupling to G proteins (see below), and, therefore, for their divergent cellular effects. In contrast, they share a high degree of homology in the seven highly conserved transmembrane domains. Based on their different affinity for selective ligands, the existence of different subtypes of ETA and ETB receptors (provisionally named ETA1, ETA2, ETA3, etc. and ETB1, ETB2, etc.) has been postulated (Table 1). However, only two genes encoding for the aforementioned two major ETA and ETB receptor subtypes exist in the mammalian genome. Thus, although a classification of ETA and ETB in pharmacologically distinguishable subtypes has been proposed, it has not gained universal acceptance because their identification at a molecular level in mammals has been elusive thus far. However, the heterogeneity of ET receptors should be taken into account in the interpretation of different results of studies that will be reviewed in this paper. Screening of amphibian libraries has revealed the existence of two additional ET receptor sub-

TABLE 1  
Main antagonists and agonists of endothelin receptors

ETA Antagonists	ETB Antagonists	Mixed ETA/ETB Antagonists	ETB Agonists
BMS-182874	BQ-788 <sup>a</sup>	PD-142-893	STX <sup>a</sup>
BMS-193884	IRL-1038 <sup>a</sup>	Ro-47-0203(Bosentan) <sup>a</sup>	BQ-1620 <sup>b</sup>
BQ-123 <sup>a,b</sup>	RES-701-1 <sup>a</sup>	SB-209670 <sup>b</sup>	IRL-3020 <sup>a</sup>
BQ-485 <sup>b</sup>	IRL-2500	SB-217242	
BQ-610 <sup>a</sup>	Ro-468443	Ro-485695	
PD-147953			
(FR-139317)			
PD-151242 <sup>a</sup>			

<sup>a</sup> BQ-123 and SB-209670 are reported to be selective for ETA<sub>1</sub> and ETB<sub>2</sub> receptor subtypes, respectively (Ohlstein et al., 1996).

<sup>b</sup> Ligands used in the studies reviewed in the present survey.

types. ETC and ETAX receptor genes were identified and cloned from octopus melanophores and heart, respectively (Karne et al., 1993; Kumar et al., 1993).

b. G PROTEIN COUPLING AND SIGNAL TRANSDUCTION. Binding of ET isopeptides to ETA and ETB heptahelical G protein-coupled receptors results in activation of at least three classes of G $\alpha$  proteins (G $\alpha_s$ , G $\alpha_i$ , and G $\alpha_q$ ; Levin, 1996a; Miyauchi and Masaki, 1999). Interestingly, different domains of the ET-receptor structure are required for the coupling of different G $\alpha$  proteins. G $\alpha_s$  protein coupling is associated to the activation of the second messenger system. G $\alpha_s$  coupling results in the activation of adenylate cyclase; G $\alpha_i$  activation leads to the inhibition of adenylate cyclase and probably the activation of voltage-gated Ca<sup>2+</sup> channels. G $\alpha_q$  coupling most likely is linked to activation of PLC and a nonselective cation channel. Other possible signaling mechanisms of ETA and ETB receptors, including PLA2 and PLD activation (for review, see Douglas and Ohlstein, 1997), will be discussed in the following sections of this review.

c. RECEPTOR ANTAGONISTS. Two approaches to inhibit the ET system have been pursued, the blockade of ET receptors with selective or mixed ETA/ETB antagonists (for review, see Ferro and Webb, 1996; Gray and Webb, 1996), and, more recently, the inhibition of ECE-1 (Turner et al., 1998). A list of the ET receptor antagonists that have been developed and/or are currently pursued as potential therapeutic agents, as well as of the main ET receptor agonists, is shown in Table 1.

### B. Steroid-Secreting Cells

Steroid-secreting cells possess common morphological features. This reflects their ability to synthesize steroid hormones starting from cholesterol, which may be taken up from circulating lipoproteins and also locally synthesized through a series of enzymes located on mitochondrial cristae and SER membranes. These cells display variously shaped mitochondria that always contain tubular or tubulovesicular cristae, a well developed SER, only a few profiles of rough endoplasmic reticulum, and a variable number of lipid droplets. The number of lipid droplets depends on the functional status of the cells because they contain cholesterol available for steroid-hormone production (for review, see Nussdorfer, 1986; Vinson et al., 1992; Sawyer, 1995; Russel, 1996).

The pathways of steroid hormone biosynthesis in the adrenal cortex and gonadal cells are depicted in Fig. 3. The first step of this process, which is selectively activated by the main agonists, is the hydrolysis of cholesterol esters stored in lipid droplets into free cholesterol, which is transported into mitochondria, where it is converted to pregnenolone by hydroxylating enzymes cleaving its side chain (P450scc). This is the rate-limiting step of steroid synthesis and is rather complex and highly regulated. To summarize, it involves the sterol carrier protein 2- and steroidogenic acute regulatory protein-

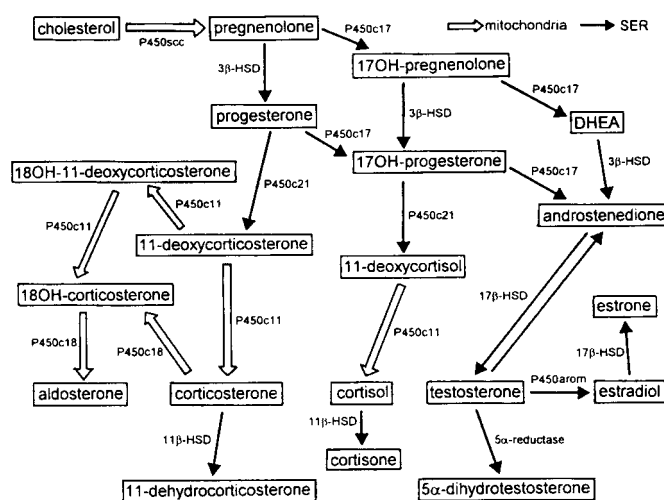


FIG. 3. Pathway of hormone synthesis and degradation in steroid-secreting cells. Steps occurring in mitochondria and SER are indicated by different arrows.

mediated transport of cholesterol to the outer mitochondrial membrane and its translocation to the P450scc located on the inner membrane, a process in which peripheral benzodiazepine receptors located on the outer mitochondrial membrane seem to play a major role (Pfeifer et al., 1993; Stocco, 1996; Stocco and Clark, 1996,1997; Papadopoulos et al., 1997; Kallen et al., 1998). Newly formed pregnenolone leaves mitochondria and reaches SER, where 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD) transforms it into progesterone. Pregnenolone and progesterone may be converted in the SER to 17-hydroxypregnenolone and 17-hydroxyprogesterone by P450c17 (17 $\alpha$ -hydroxylase). At this point, the pathway of steroid synthesis differs in various steroid-secreting cells, i.e., adrenocortical cells, testis Leydig cells, granulosa and thecal cells of ovarian follicles, and lutein cells (either of granulosa or thecal origin; for review, see Erickson et al., 1985; Nussdorfer, 1986; Amsterdam and Romensch, 1987; Miller, 1988; Niswender and Nett, 1988; Fraser, 1992; Hanukoglu, 1992; Gibori, 1993; Saez, 1994; Sawyer, 1995; Payne and O'Shaughnessy, 1996; Boon et al., 1997).

1. Adrenocortical Cells. P450c21 (21-hydroxylase), located in the SER, converts progesterone and 17-hydroxyprogesterone to 11-deoxycorticosterone and 11-deoxycortisol, respectively. These two steroids penetrate the mitochondria, where P450c11 (11 $\beta$ -hydroxylase) transforms them into corticosterone and cortisol, which are the glucocorticoids produced by the zona fasciculata/reticularis (ZF/R) of rodents and other mammals, respectively. P450c11 also possesses 18-hydroxylase activity, thereby producing little amounts of 18-hydroxy-11-deoxycorticosterone and 18-hydroxy-11-deoxycortisol. In these zones, and especially in the zona reticularis (ZR), a P450c17-coupled C17,20-lyase located in SER may convert 17-hydroxypregnenolone and 17-hydroxyprogesterone into adrenal androgens, i.e., dehydroepiandrosterone (DHEA)

and androstenedione. In the zona glomerulosa (ZG), corticosterone is converted to 18-hydroxycorticosterone and then aldosterone by intramitochondrial P450c18 (a mixed hydroxylase-dehydrogenase, also named aldosterone synthase). Of interest, mammalian adrenocortical cells also express the 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ -HSD) gene and possess 11 $\beta$ -HSD activity (Yang and Matthews, 1995; Musajo et al., 1996; Roland and Funder, 1996; Shimajo et al., 1996; Smith et al., 1997; Mazzocchi et al., 1998c). This enzyme, located in the SER (Náray-Fejes-Toth et al., 1998), catalyzes the conversion of the glucocorticoids cortisol and corticosterone into their corresponding inactive forms, cortisone and 11-dehydrocorticosterone, respectively (for review, see Funder, 1995, 1996). Evidence has been provided that indicates that 11 $\beta$ -HSD activity is negatively modulated by the rise in the intra-adrenal concentration of non-11 $\beta$ -hydroxylated steroid hormones (Musajo et al., 1996; Morita et al., 1997; Mazzocchi et al., 1998c). The main agonists involved in the physiological regulation of the secretory activity of adrenocortical cells are adrenocorticotrophic hormone (ACTH), ANG-II, and K<sup>+</sup> (for review, see Quinn and Williams, 1992; Vinson et al., 1992; Ganguly and Davis, 1994). ACTH stimulates both ZG and ZF/R cells, acting through specific receptors mainly coupled with the adenylate cyclase/PKA-dependent signaling pathway. ANG-II stimulates zona glomerulosa cells, acting via AT1 receptors mainly coupled with PLC-dependent cascade. PLC catalyzes the breakdown of phosphatidylinositol to inositol-triphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). DAG activates PKC, and IP<sub>3</sub> enhances Ca<sup>2+</sup> release from intracellular stores, thereby raising intracellular (cytosolic) calcium concentration ([Ca<sup>2+</sup>]<sub>i</sub>), which in turn activates PKC. There is evidence that ANG-II may also activate tyrosine kinase (TK)-dependent cascade. In humans and calves, ANG-II is also able to stimulate ZF/R cells secretion (Bird et al., 1992; Ouali et al., 1992; Clyne et al., 1993; Lebrethon et al., 1994). There is general agreement that ACTH and ANG-II are able to activate both the cyclooxygenase (CO) and lipoxygenase (LO) pathways of arachidonic acid, leading to the production of prostaglandins (PGs) and 12-hydroxyeicosatetraenoic acid, respectively. Arachidonic acid may be released from plasma membrane phospholipids by the action of PLA2 or it may derive from DAG. K<sup>+</sup> electively depolarizes plasma membrane of ZG cells, thereby opening voltage-gated Ca<sup>2+</sup> channels and raising [Ca<sup>2+</sup>]<sub>i</sub>. The main pathways involved in the signaling mechanism of steroid-secreting-cell agonists and their possible interrelationships are depicted in Fig. 4, and the most widely employed selective stimulators and inhibitors of the various steps of the signaling cascades are shown in Table 2.

**2. Leydig Cells.** 17-Hydroxypregnenolone and 17-hydroxyprogesterone are converted into DHEA and androstenedione by P450c17/17-20-lyase, as occurs in adrenal ZR. DHEA, in turn, is transformed to androstenedione by 3 $\beta$ -HSD. A 17 $\beta$ -HSD, located in the SER, then transforms androstenedione to testosterone, the main andro-

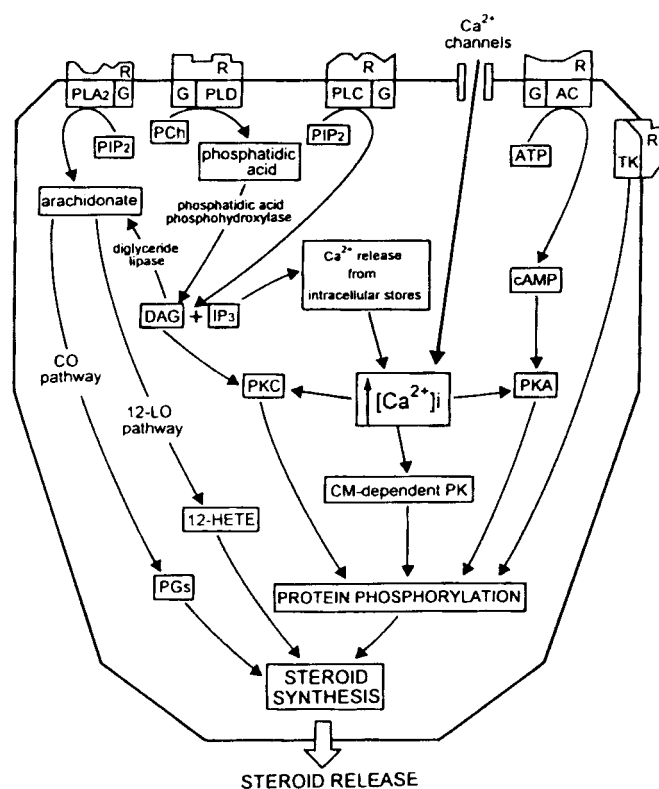


FIG. 4. Main pathways in the signaling mechanisms of agonists in steroid-secreting cells. AC, adenylate cyclase; CM, calmodulin; G, G protein; PCh, phosphatidyl choline; PIP<sub>2</sub>, phosphatidylinositol biphosphate; R, receptor. Other abbreviations are indicated in the text.

gen produced by the testis. Testosterone may be partially inactivated by 5 $\alpha$ -reductase, which transforms it into 5 $\alpha$ -dihydrotestosterone, or in small amounts aromatized to estradiol by P450arom. All of these last steps occur in the SER. Luteinizing hormone (LH) is absolutely required for the maintenance of Leydig cell-specific functions and is the main secretagogue of testosterone, at least under physiological conditions (for review, see Saez, 1994). LH acts through specific LH/human chorionic gonadotropin (hCG) G protein-coupled receptors, the main signaling mechanism of which is the activation of adenylate cyclase/PKA cascade. Evidence also is available that PLC- and PLA2-dependent cascades may play a role in transducing LH secretagogue signals (for review, see Cooke, 1996; Fig. 4).

**3. Granulosa and Thecal Cells and Lutein Cells.** The pathway of steroid synthesis, leading to the production of progesterone and estrogens, is the same as described above and is strictly controlled by follicle-stimulating hormone (FSH) and LH. Again, progesterone may be inactivated by 20 $\alpha$ -HSD and 5 $\alpha$ -reductase. Granulosa cells are exclusively provided with FSH receptors, whereas thecal cells (located in the theca interna) possess LH-hCG receptors, both receptors being mainly coupled with the adenylate cyclase/PKA signaling cascade (Fig. 4). Thecal cells under the influence of LH are stimulated to differentiate and produce androstenedione and



TABLE 2  
Main inhibitors and activators of the signaling cascades

Adenylate cyclase/PKA-dependent cascade	
Forskolin <sup>a</sup> .....	adenylate cyclase activator
SQ.2253 .....	adenylate cyclase inhibitor
H-89 <sup>a</sup> .....	selective PKA inhibitor
PLC/PKC-dependent cascade	
U-73122 <sup>a</sup> .....	selective PLC inhibitor
PMA <sup>a</sup> .....	PKC activator
Staurosporin <sup>a</sup> .....	nonselective PKC inhibitor
Ro-31-8220 <sup>a</sup> .....	selective PKC inhibitor
Calphostin-C .....	selective PKC inhibitor
PLA2/CO- or LO-dependent cascade	
Quinacrine <sup>a</sup> .....	PLA2 inhibitor
AACOF <sub>3</sub> .....	selective PLA2 inhibitor
Indomethacin <sup>a</sup> .....	CO inhibitor
Nordihydroguaiaretic acid <sup>a</sup> .....	LO inhibitor
Phenidone .....	LO inhibitor
PLD-dependent cascade	
Propranolol .....	PLD inhibitor
TK-dependent cascade	
Tyrphostin-23 <sup>a</sup> .....	selective TK inhibitor
Genistein .....	selective TK inhibitor
Calmodulin-dependent cascade	
CaM kinase II (290–309) .....	calmodulin antagonist
Calmidazolium (R24571) .....	calmodulin antagonist
W-5 .....	calmodulin antagonist
Ca <sup>2+</sup> channels	
BAYK-8644 <sup>a</sup> .....	selective L-type Ca <sup>2+</sup> channel activator
Nicardipine <sup>a</sup> .....	selective L-type Ca <sup>2+</sup> channel blocker
Verapamil <sup>a</sup> .....	selective L-type Ca <sup>2+</sup> channel
Amiloride .....	T-type Ca <sup>2+</sup> channel blocker

<sup>a</sup> Chemicals used in the studies reviewed in the present survey.

little amounts of testosterone. These hormones diffuse through the basement lamina, reaching granulosa cells, which, under the influence of FSH, aromatize them to estradiol and estrone. Estrogens diffuse into the capillaries of the theca interna or are poured in the intracellular spaces of the granulosa. The rise in the level of circulating estrogens results in the preovulatory surge of LH, and the increasing intrafollicular concentration of estrogens stimulates proliferation of granulosa cells, which in the preovulatory period acquire LH receptors. Under the influence of LH, the production of estrogens of granulosa cells declines, and their main secretory product becomes progesterone. After ovulation, steroid-secreting cells of the ovarian follicle differentiate into granulosa lutein cells and thecal lutein cells, which both secrete large amounts of progesterone, the elevated levels of LH greatly enhancing in them the expression of P450<sub>scc</sub> and 3β-HSD genes (for review, see Erickson et al., 1985; Hanukoglu, 1992; Hsueh and Billig, 1995).

### III. Endothelin Biosynthesis in Steroid-Secreting Tissues

Much evidence indicates that ETs are synthesized in the mammalian adrenal gland, testis, and ovary. At present, no data are available on this matter as far as lower vertebrates are concerned.

### A. Gene Expression Studies

1. *Adrenal Gland.* Following the preliminary findings of low-abundance expression of ET-1 mRNA in the porcine adrenal gland (Nunez et al., 1990), numerous studies were carried out in tissues harvested from humans and rats that used reverse transcription-polymerase chain reaction (RT-PCR) and Southern hybridization with a 21-mer <sup>35</sup>S-labeled oligonucleotide probe complementary to a sequence within the cDNA between the primers.

a. *HUMAN ADRENALS.* The presence of ppET-1 mRNA was first demonstrated in homogenates of adrenals from patients with aldosterone-producing adenomas (APA) causing Conn's syndrome who were treated by adrenalectomy (Imai et al., 1992). Using Northern blotting of poly(A)<sup>+</sup> RNA and hybridization with a specific cDNA probe for the ET-1 gene, ppET-1 mRNA was detected in the normal cortical tissue surrounding the tumor. These results were confirmed by Rossi et al. (1994, 1995b), who studied the ppET-1 gene expression by RT-PCR on homogenates of a large number of normal adrenals obtained from patients undergoing unilateral adrenalectomy for kidney cancer. However, the use of tissue homogenates prevented definitive conclusions concerning the parenchymal or extraparenchymal location of the ppET-1 gene, and the same was true for the ECE-1 gene, whose expression was demonstrated in homogenates of both adrenocortical and adrenomedullary tissues (Rossi et al., 1995a). To address this issue, Rossi et al. (1997b) used dispersed and purified adrenocortical cells. RT-PCR evidenced the expression of ppET-1 and ECE-1 genes in these preparations, thereby making it likely that human adrenocortical cells are able to synthesize ET-1. These findings were confirmed by Mathieu et al. (1988) by using primary cultures of human adrenal cells enriched in ZG cells. Although ETs are known to play an important role in the development of neural crest-derived cells (Kurihara et al., 1994; Brand et al., 1998) and ET mRNAs have been detected in pheochromocytomas (Amico et al., 1993; Kon, 1993), ET genes do not appear to be expressed in normal adrenal medulla.

b. *RAT ADRENALS.* The expression of the ppET-1 gene was demonstrated by RT-PCR in both homogenates and dispersed ZG or ZF/R cells (Belloni et al., 1996c). The presence of ET gene products in adrenal medulla has not been reported.

2. *Testis.* ET-1 protein and ppET-1 mRNA have been detected in homogenates of the rat testis (Matsumoto et al., 1989; Sakurai et al., 1991). This finding was confirmed in the human testis by in situ hybridization (Er-gun et al., 1998).

3. *Ovary.* a. *PRIMATE OVARY.* ppET-1 mRNA has been demonstrated by Northern blot analysis in the ovarian homogenates. Subsequent in situ hybridization showed its presence in the granulosa cells, but not in the thecal compartment of follicles at different stages of maturation.

tion (Magini et al., 1996). The hybridization signal was especially prominent in the granulosa cells of the tertiary follicles, and also detectable in the endothelial cells of the blood vessels. Western blot analysis revealed the presence of ECE-1 in the menstrual corpora lutea, the enzyme being more abundant in early and midluteal phases than in the late luteal phase (Yoshioka et al., 1998). Karam et al. (1999) confirmed the presence of ECE-1 gene expression in the theca interna cells of secondary, tertiary, and atretic follicles, as well as in the corpora lutea of human and monkey ovary; however, ET-1 mRNA was observed only in the endothelial cells of blood vessels. The presence of ET-like immunoreactivity (LI) in the follicular fluid has been studied by several groups. Earlier radioimmunoassay measurements gave rather inhomogenous results, with ET-1 concentration ranging from 12 to 200 fmol/ml in mature follicles, and from 0.2 to 0.4 pmol/ml in immature ones (Kamada et al., 1993b; Abae et al., 1994; Kubota et al., 1994). However, an inverse correlation between ET-1 concentration and the maturity of follicles has not been confirmed by other investigators (Schiff et al., 1993; Magini et al., 1996; Sudik et al., 1996). Haq et al. (1996) reported the presence of ET-1-LI, ET-2-LI and big-ET-1-LI in the follicular fluid obtained from mature follicles at the time of oocyte aspiration in women undergoing ovulation induction by human menopausal gonadotropin. The mean concentrations of the three peptides were 6.8, 12.6, and 8.2 fmol/ml, respectively. Sudik et al. (1996) studied the level of ET-1 and ET-2 in follicular fluids of 57 women undergoing an in vitro fertilization-embryo transplantation (IVFET) program. ET-1-LI was detected in all samples ( $7.6 \pm 4.8$  fmol/ml), and ET-2-LI in about 68% of samples ( $5.5 \pm 6.3$  fmol/ml). A significant negative correlation existed between ET-2 concentration and follicle size. No obvious correlation was observed between ET concentration and estradiol, progesterone, and testosterone content in the follicular fluid (Kamada et al., 1993b; Schiff et al., 1993; Sudik et al., 1996). These findings strongly suggest the involvement of ETs in the follicular development. In keeping with this contention are the observations of Stones et al. (1996), who studied ET release from isolated perfused premenopausal ovaries. Of the 12 studied, five released ETs, and four of these contained either a developing follicle or a corpus luteum. Seven of the eight inactive ovaries did not release ETs.

b. CALF OVARY. Northern blot hybridization revealed the expression of ET-1 gene in ovary homogenates (Fujii et al., 1991). ET-1 mRNA and protein levels in the bovine corpora lutea depended on the stage of estrous cycle (Girsh et al., 1996a,b). In young corpora lutea (day 5) they were  $2.9 \pm 0.3$  U and  $6.8 \pm 1.2$  fmol/mg, respectively. In old corpora lutea (day 18)  $7.4 \pm 0.5$  U and  $19.5 \pm 1.9$  fmol/mg. Accordingly, plasma ET-1 concentration increased around luteolysis and estrus (Ohtani et al., 1998).

c. PIG OVARY. The expression of ppET-1 mRNA and the presence of ET-1-LI have been demonstrated in cultured granulosa cells from small or medium follicles (Kamada et al., 1993a; Kubota et al., 1994). The concentration of ET-1-LI in the follicular fluid was 3.6 to 5.7 fmol/ml (Iwai et al., 1991).

d. RAT OVARY. The ET-1 content in the ovary of adult animals was very elevated ( $1.2 \pm 0.2$  pmol/g; Usuki, 1991; Usuki et al., 1991c). High concentrations of ET-1-LI have been also detected in the corpora lutea of superovulated rats and follicles (Usuki et al., 1991b, c, 1992). The fact that these concentrations were higher than those present in the blood indicates the local synthesis of ETs.

### B. Immunocytochemical Studies

The presence of immunoreactive ETs in steroid-secreting tissues has been investigated by the use of specific polyclonal and monoclonal antibodies against ETs risen in different species (Kondoh et al., 1990; Traish et al., 1992).

1. *Adrenal Gland.* All of the studies were carried out in normal or pathologic human glands. Li et al. (1994) reported the presence of ET-1-LI especially in the ZF, where about 50% of parenchymal cells were positive. Immunostained cells were few in the ZG and ZR, and absent in the vascular elements of the gland. Immunostaining appeared in the form of grains, vacuoles, or membranes. Subsequent electron microscopic studies (Li et al., 1995a) showed that vacuoles and grains corresponded to ET-1 located around lipid droplets and in endoplasmic reticulum-mitochondria, respectively, whereas ET-1 found in membranes was probably bound to its receptors. More recently, Li et al. (1999) confirmed the distribution of ET-1-positive cells in the human adrenal, but reported that the extracted ET-1-like protein had a molecular mass of 9 kDa, i.e., lower than that of ppET-1 (21 kDa) and higher than that of ET-1 (2.5 kDa). The functional relevance of this finding remains to be elucidated, although it could suggest alternative post-translational processing of big-ET-1 in the human adrenals (see *Section IIA*). According to Hiraki et al. (1997), ET-1/big-ET-1-LI was present throughout all of the cortex, although more abundant in the ZF, with ET-3/big-ET-3-LI being very limited in the cortical cells. All of these investigators agree that ET-LI was absent in adrenal medulla, although ET-1-LI and ET-3-LI were detected in pheochromocytomas (Sone et al., 1991; Watanabe et al., 1997). Contrasting findings were obtained by Davenport et al. (1996,1998), who reported ET-1/big-ET-1-LI and ECE-1-LI to be exclusively confined to endothelial cells of the smaller resistance vessels of the pericapsular arterial plexus and the central vein, and ET-3/big-ET-3-LI in the adrenal medulla.

2. *Testis.* According to Fantoni et al. (1993), in 20-day-old rats, Leydig cells did not contain immunocytochemically detectable ET-1-LI, whereas Sertoli cells were positive. ET-1-LI was detectable in Leydig cells, as well as

in Sertoli and endothelial cells, of adult rat testis (Fantoni et al., 1993; Collin et al., 1996).

3. *Ovary*. The presence of immunostaining for ET-1 in the human ovarian cortex has been demonstrated by Magini et al. (1996). ET-1-LI was located in the wall of follicles at different stages of maturation, especially in the cytoplasm of granulosa cells and in the endothelial cells of the thecal capillaries. Follicles at the early stage of development were deprived of ET-1-LI. In contrast, Karam et al. (1999) reported the presence of ET-1-LI exclusively in the blood vessels of the human and monkey ovaries. ECE-1 was immunocytochemically detected in the human ovary (Yoshioka et al., 1998): in the pre-ovulatory follicles, immunostaining was weak in granulosa cells and moderate in theca interna cells; in both menstrual and pregnant corpora lutea, abundant immunostaining was contained in both types of lutein cells, thereby suggesting that ECE-1 expression increases during luteinization.

### C. Summary

RT-PCR demonstrated the expression of the ET-1 and ECE-1 genes in the mammalian adrenal glands and ovary (developing corpora lutea), and of the ET-1 gene in the Leydig cells of the testis. Radioimmunoassay and immunocytochemistry detected ET-LI in all three steroidogenic tissues.

## IV. Endothelin Receptor Subtypes in Steroid-Secreting Tissues

The gene expression, localization, and binding properties of ETA- and ETB-receptor subtypes have been investigated only in steroidogenic tissues of mammals by both biochemical and morphological techniques.

### A. Gene Expression Studies

1. *Adrenal Gland*. a. HUMAN ADRENALS. The expression of the ET receptor mRNAs was first detected by Northern blotting analysis in homogenates of normal cortical tissue surrounding APAs. However, the results of the <sup>125</sup>I-ET-1 competitive displacement binding were consistent with the presence of only a single class of receptors (Imai et al., 1992). The presence of both ETA- and ETB-subtype mRNAs was conclusively demonstrated by Rossi et al. (1994, 1995b). They were able to detect by RT-PCR the expression of both receptor-subtype genes in homogenates of a large number of histologically normal adrenal cortices of patients adrenalectomized for kidney cancer. These findings were subsequently confirmed by Davenport et al. (1996). Further studies showed the presence of both ETA- and ETB-receptor mRNAs in isolated and purified normal human adrenocortical cells (Rossi et al., 1997b).

b. RAT ADRENALS. ET-receptor mRNAs were detected by in situ hybridization by Hori et al. (1992). The hybridization signal for ETB mRNA was diffusely distributed in both cortex and medulla, whereas that for ETA

mRNA was almost exclusively restricted to the corticomedullary border, in the blood vessels of this region. To localize the ETA and ETB genes in the different adrenocortical zones, Belloni et al. (1996c) investigated by RT-PCR both homogenates and dispersed ZG and ZF/R cells. Although they found the specific mRNA for both receptor subtypes in tissue homogenates, they could detect only the ETB-receptor mRNA in both dispersed adrenocortical-cell types. This last finding strongly suggested the extraparenchymal expression of the ETA gene in the adrenal cortex of this species.

2. *Testis*. Maggi et al. (1995) detected by Northern blot analysis the specific mRNAs for ETA and ETB receptors in homogenates of human testis, with the level of ETA expression being about 7-fold higher than that of ETB, a finding consistent with the reported lack of ETB-receptor mRNA in the rat testis (Sakurai et al., 1990).

3. *Ovary*. In cultured luteinized human granulosa cells obtained from patients undergoing an IVFET program, Northern blot analysis revealed the expression of both ETA- and ETB-receptor genes. The ETA-receptor mRNA was much more abundant than that of ETB receptors (Kamada et al., 1995). However, in situ hybridization studies showed the presence of both ETA- and ETB-receptor mRNAs only in the vascular component of the human and monkey ovaries (Karam et al., 1999). The mRNAs of both ET-receptor subtypes were detected in the rat ovary, but in this case, ETB expression prevailed over that of ETA (Iwai et al., 1993). In situ hybridization showed that ETB mRNA was abundant in the granulosa cells of developing follicles and absent in atretic and preantral ones. No hybridization signals were observed in the thecal cells.

### B. Saturation and Inhibition Binding Studies

1. *Adrenal Gland*. a. HUMAN ADRENALS. Earlier studies showed that in human adrenocortical membranes, <sup>125</sup>I-ET-1 saturation isotherms attained an equilibrium after 30 min at 30°C; competitive displacement by ET-2 and ET-3 was consistent with the presence of a single class of high-affinity binding sites (Nunez et al., 1990). However, different findings were obtained by Rossi et al. (1994), who were able to demonstrate the presence of both ETA- and ETB-receptor subtypes, and to characterize their density and binding properties.

b. CALF ADRENALS. In bovine ZG cell cultures, <sup>125</sup>I-ET-1 binding was time-dependent, was saturable, and reached an apparent equilibrium after 60 min at room temperature (Cozza et al., 1989). Scatchard analysis revealed the presence of a single class of high-affinity binding sites with an apparent  $K_d$  for ET-1 of 100 pM and a  $B_{max}$  of 52,500 receptors/cell. ET-3 was only about 40% as potent as ET-1. Further investigation (Gomez-Sanchez et al., 1990) showed that although ET-1 and STXb were equipotent in their aldosterone secretagogue action on ZG cell cultures, STXb displaced <sup>125</sup>I-ET-1 binding with only 3% of the ET-1 potency. This discrep-

ancy suggested the possibility of the presence of a second adrenal receptor for ET-1, a hypothesis that was further supported by the observation that STXB was more effective than ET-1 in down-regulating the higher-affinity, lower-capacity binding site.

c. RAT ADRENALS. A high density of  $^{125}\text{I}$ -ET-1 binding sites, which was surpassed only by that found in the lung, was reported in the adrenal gland by both in vitro and in vivo studies, with the adrenal medulla being much more heavily labeled than the cortex (Koseki et al., 1989a,b). More recent investigations (Kapas et al., 1996) demonstrated  $^{125}\text{I}$ -ET-1 binding in the cortex, which was saturable at from 75 to 120 and from 175 to 200 pM in the ZG and ZF/R, respectively. Scatchard analysis revealed the presence of two distinct populations of binding sites in the ZG: high-affinity ( $K_d$ , 1.87 nM;  $B_{\max}$ , 535 fmol/ $10^6$  cells) and low-affinity ( $K_d$ , 10.25 nM;  $B_{\max}$ , 1047 fmol/ $10^6$  cells) binding sites; conversely, ZF/R contained only a single class of low-affinity binding sites ( $K_d$ , 4.95 nM;  $B_{\max}$ , 521 fmol/ $10^6$  cells). BQ-123 inhibited  $^{125}\text{I}$ -ET-1 binding in the ZG with a potency comparable to that of ET-1, whereas RES-701-1 was less effective; the reverse occurred in the ZF/R. Kapas et al. (1996) concluded that the ETA receptors predominate in rat ZG and ETB receptors in the inner cortical zones.

2. *Testis*. The presence of a single class of ET binding sites has been described in rat testis homogenates (Sakaguchi et al., 1992) and dispersed Leydig cells (Fantoni et al., 1993). Scatchard analysis demonstrated a  $B_{\max}$  of 250 and 13 pmol/mg of protein, respectively. Competition curves showed the following rank of affinity: ET-1 = ET-2 ( $K_d$ , 0.6 nM)  $\gg$  ET-3 ( $K_d$ , 6 nM)  $\gg$  big-ET-1, thereby suggesting that the binding sites were ETA receptors. A single class of ET-1 binding sites ( $K_d$ , 1 nM;  $B_{\max}$ , 59 fmol/ $10^6$  cells) was also reported in the murine tumor cell line MA-10 (Ergul et al., 1993).

3. *Ovary*. a. HUMAN OVARY. Two distinct populations of ET-binding sites were found in the human ovary, with the following binding parameters:  $B_{\max}$ , 5309 versus 50 fmol/mg of protein;  $K_d$  for ET-1, 0.88 versus 0.02 nM;  $K_d$  for ET-3, 344 versus 0.01 nM;  $K_d$  for BQ-123, 5 versus  $>10,000$  nM; and  $K_d$  for IRL-1620,  $>10,000$  versus 14 nM. These data clearly indicated that the two classes of binding sites were ETA and ETB receptors, respectively, ETA being about 100-fold more abundant than ETB (Mancina et al., 1997).

b. CALF OVARY. A single class of high-affinity ET binding sites has been demonstrated in cultured lutein cells by Girsh et al. (1996a). Scatchard analysis indicated a  $B_{\max}$  of from 38 to 50 fmol/ $10^5$  cells, and a  $K_d$  for ET-1 of from 0.2 to 0.5 nM. The  $K_d$  for ET-3 was 3 orders of magnitude less. This finding, along with the selective displacement of  $^{125}\text{I}$ -ET-1 binding by BQ-123, suggested that the binding sites were ETA receptors. However, the inhibition curve of ET-1 binding by ET-3 was biphasic in the whole corpus luteum-derived cells, probably due to the presence of ETB receptors in the endothelial cells.

c. PIG OVARY. The presence of a single class of high-affinity binding sites for both ET-1 and ET-3 was demonstrated in cultured granulosa cells (Kamada et al., 1992) and membranes of granulosa cells harvested from small- or medium-sized follicles (Kubota et al., 1994):  $B_{\max}$  and  $K_d$  were 1.84 pmol/mg of protein and 0.6 nM, respectively. Similar results were obtained more recently by Flores et al. (1995). All of the investigators agree that these binding sites are predominantly ETA receptors.

d. RAT OVARY. In dispersed or cultured granulosa cells obtained from immature rat ovaries, a single class of high-affinity ET binding sites was found, which displayed similar affinity for ET-1, ET-2, and ET-3 (Tedeschi et al., 1994; Otani et al., 1996). Scatchard analysis demonstrated a  $B_{\max}$  of  $2.5 \text{ fmol} \times 10^5 \text{ cell}$  and a  $K_d$  of 0.23 nM. Otani et al. (1996) also showed that  $^{125}\text{I}$ -ET-1-specific binding was concentration-dependently increased by FSH, decreased by LH, and unaffected by  $10^{-7}$  M estradiol, androstenedione, or progesterone. A promoting effect of FSH on ET-1 binding of granulosa cells was also observed by Usuki et al. (1998). Subsequent equilibrium-binding studies with  $^{125}\text{I}$ -Tyr<sup>13</sup>-ET-1 and the ETB receptor agonist  $^{125}\text{I}$ -IRL-1620 provided evidence that rat granulosa cells possessed both ETA and ETB receptors, and that FSH predominantly increased the number and affinity of the ETA subtype.

### C. Immunocytochemical Studies

The lack of widely available antibodies specific for each ET-receptor subtype until now has prevented extensive investigations.

1. *Adrenal Gland*. Hagiwara et al. (1993) studied the localization of the ETB receptor in the bovine adrenals and found prominent staining exclusively in the endothelial cells of inner zones. More recently, using antibodies against ETA (amino acid residues 59–69) and ETB (residues 420–433) raised in rabbits, Hiraki et al. (1997) observed the presence of ETB receptors especially in the ZG of the bovine adrenal cortex; whole ETA immunostaining was only occasionally seen in the cortex. The adrenal medulla did not show any staining with either antiserum. The discrepancy of these findings with the distribution of ETA- and ETB-receptor mRNAs may be explained by assuming that ET receptor density is not high enough to be visualized with this technique.

2. *Testis*. Using a specific antiserum raised against ETB receptors, Hagiwara et al. (1993) showed that in the bovine testis, the percentage of immunoprecipitable ET-1 binding sites was only 5%, thereby making it likely that 95% of binding sites were ETA receptors. However, the presence of both ETA and ETB receptors has been reported recently in human Leydig cells (Ergun et al., 1998).

3. *Ovary*. The exact location of ET-receptor subtypes in the ovarian follicles and corpus luteum has not been demonstrated by immunocytochemistry.

### D. Autoradiographic Studies

1. *Adrenal Gland.* a. HUMAN ADRENALS. Using in vitro labeling and quantitative densitometry, Davenport et al. (1989) showed that  $^{125}\text{I}$ -ET-1 binding was about 2-fold higher in the outer cortex than in the ZF/R. The kind and distribution of ETA- and ETB-receptor subtypes has been investigated by the use of selective ligands (Belloni et al., 1994; Rossi et al., 1994). Total  $^{125}\text{I}$ -ET-1 ( $10^{-9}$  M) binding was intense in the ZG, whereas in the inner cortex it appeared weak and mainly confined between parenchymal-cell cords; the muscular wall of the extracapsular arterioles was heavily labeled. BQ-123 ( $10^{-7}$  M) eliminated labeling in the vessels and markedly decreased it in the ZG without affecting binding in the ZF; in contrast, BQ-788 and STXc ( $10^{-7}$  M) lowered labeling in the ZG and completely inhibited it in the ZF without changing binding in the capsular vessels. Collectively, these findings allowed these investigators to conclude that ZG was provided with both ETA and ETB receptors, and ZF with only ETB receptors. More recently, Davenport et al. (1996) labeled ETA and ETB receptors using  $^{125}\text{I}$ -PD-151242 and  $^{125}\text{I}$ -BQ-3020, respectively, and performed saturation-binding assay with computer-assisted densitometric analysis of autoradiograms. ETA receptors were present only in the ZG ( $K_d$ , 140 pM;  $B_{\max}$ , 70 fmol/mg of protein). In contrast, ETB receptors were found throughout the entire gland: ZG ( $K_d$ , 101 pM;  $B_{\max}$ , 63 fmol/mg of protein), ZF ( $K_d$ , 145 pM;  $B_{\max}$ , 68 fmol/mg of protein), ZR ( $K_d$ , 118 pM;  $B_{\max}$ , 72 fmol/mg of protein), and the adrenal medulla ( $K_d$ , 145 pM;  $B_{\max}$ , 76 fmol/mg of protein).

b. RAT ADRENALS. Earlier studies showed that, like in humans,  $^{125}\text{I}$ -ET-1 labeling in rats was higher in the outer than the inner adrenal layers (Davenport et al., 1989; Kohzuki et al., 1989, 1991). Kohzuki et al. (1991) also calculated by quantitative densitometry a  $K_a$  for ET-1 binding of 7.1 and  $9.5 \times 10^{-9}$  M in the ZG and the adrenal medulla, respectively. Although Koseki et al. (1989a,b) reported heavier labeling of the medulla than of the cortex after in vivo i.v. injection of  $^{125}\text{I}$ -ET-1, Neuser et al. (1989, 1991), using whole-body autoradiography, showed about equal  $^{125}\text{I}$ -ET-1 enrichments in the two zones. A diffuse labeling of the entire gland was also observed after the injection of  $^{125}\text{I}$ -big-ET-1 (Neuser et al., 1991). The use of selective ligands of ETA and ETB receptors and computer-assisted densitometry allowed Belloni et al. (1995, 1996c) to show that rat adrenal possessed the same receptor-subtype distribution as human glands. Subsequent studies (Belloni et al., 1997) not only confirmed the previous results, but also demonstrated that  $^{125}\text{I}$ -ET-3 and  $^{125}\text{I}$ -BQ-3020 binding was present in both the cortex and the medulla and was completely displaced by BQ-788, but not by BQ-123. Taken together, the above surveyed findings clearly indicate the presence of both ETA and ETB receptors in

the ZG and the adrenal medulla, and of only ETB receptors in the ZF/R.

2. *Testis.*  $^{125}\text{I}$ -ET-1 binding sites were autoradiographically demonstrated in Leydig and peritubular myoid cells of the rat testis (Sakaguchi et al., 1992; Fantoni et al., 1993; Maggi et al., 1995; Belloni et al., 1996a). Binding of Sertoli cells and seminiferous tubules was very scarce. Belloni et al. (1996a) provided evidence that  $^{125}\text{I}$ -ET-1 binding was displaced by BQ-123 and unaffected by BQ-788 and STXc, thereby confirming that Leydig cells were almost exclusively provided with ETA receptors.

3. *Ovary.* In situ  $^{125}\text{I}$ -ET-1- and  $^{125}\text{I}$ -ET-3-binding inhibition studies showed that the majority of ETA and ETB receptors were present in the blood vessels of the human ovary. ETA receptors were located in the theca interna cells of the ovulatory follicles, in close proximity to the granulosa layer, the cells of which were weakly labeled. ETB receptors were absent in human follicles (Mancina et al., 1997).  $^{125}\text{I}$ -ET-1 binding sites were autoradiographically demonstrated in the granulosa cell layer of preovulatory follicles and in the vascular component of corpora lutea of the pig ovary; binding was absent in the granulosa cells of preantral follicles and in lutein cells (Flores et al., 1995). Displacement binding studies with selective ligand indicated that ET receptors were of the ETA subtype. Cultured granulosa cells collected from large antral follicles retained their ET-1 binding capacity at a higher rate than that of cells obtained from immature follicles (Flores et al., 1995). In the pregnant mare serum- and hCG-treated rat ovaries,  $^{125}\text{I}$ -ET-1 binding sites were exclusively detected in the granulosa cells, especially of the antral follicles. They were absent in the thecal and lutein cells, as well as in the atretic follicles (Otani et al., 1996). Mancina et al. (1997) confirmed this observation and showed that ET binding sites were ETB receptors by using selective ligands.

### E. Summary

RT-PCR demonstrated the expression of both ETA- and ETB-receptor genes in the mammalian adrenal glands. The expression of ETA gene prevails over that of ETB gene in the testis and ovary. Saturation- and inhibition-binding studies showed that adrenal glands are provided with two classes of ET receptors, whereas testis and ovary almost exclusively possess ETA receptors. Immunocytochemical and radioautographic studies confirmed these findings, and additionally demonstrated that adrenal ZG and medulla contain both ETA and ETB receptors, and inner adrenocortical zones contain only ETB receptors.

## V. Effects of Endothelins on the Secretory Activity of Steroid-Secreting Cells

### A. Hormone Secretion

1. *Adrenal Cortex.* A potent secretagogue action of ETs has been firmly demonstrated on both mineralocorticoid and glucocorticoid hormones.

a. HUMAN ADRENOCORTICAL CELLS. By using a mixture of dispersed human ZG and ZF/R cells, Hinson et al. (1991c) observed a marked stimulatory action of both ET-1 and ET-3 on basal aldosterone and cortisol secretions. Minimal effective concentrations were  $10^{-14}$ / $10^{-13}$  M. The maximal effective concentration was  $10^{-7}$  M, which elicited 5- and 11- to 30-fold rises in aldosterone and cortisol, respectively. These findings with ET-1 were confirmed recently by studies showing that  $10^{-9}$  M ET-1 elicited 5- and 3-fold increases in aldosterone and cortisol output (Rossi et al., 1997b). Moreover, these authors showed a marked pregnenolone secretagogue effect of ET-1, thereby indicating that the peptide enhanced both early and late steps of steroid synthesis (Fig. 3). Less-marked stimulatory effects of ET-1 were observed by using adrenal slices obtained from kidney donors or fragments of normal adrenal tissue adjacent to APAs (Zeng et al., 1992). In those tissues, aldosterone secretion was increased, with minimal and maximal effective concentrations of  $10^{-11}$  M (30% rise) and  $10^{-8}$  M (60% rise), respectively. In vivo studies carried out in human volunteers did not show any effect of ET-1 infusion on both plasma renin activity (PRA) and basal aldosterone or cortisol plasma concentrations (Vierhapper et al., 1990, 1995). However, the i.v. infusion of ET-1, at a rate of 2 pmol/kg · min for 15 min and 1 pmol/kg · min for the subsequent 105 min, evoked a 75% potentiation of plasma aldosterone response to the bolus injection of 0.25 mg ACTH.

b. BOVINE ADRENOCORTICAL CELLS. Using cultured bovine ZG and ZF cells, Rosolowsky and Campbell (1990) were unable to observe any effect of ET-1 on either basal aldosterone and cortisol secretion or ANG-II-stimulated aldosterone release over a range of concentrations from  $10^{-11}$  to  $10^{-6}$  M. However, they showed a clear-cut potentiating action of ET-1 on both ACTH- and dibutyryl (db) cAMP (cAMP)-stimulated aldosterone release. The maximal effective concentration was  $10^{-8}$  M (about 50% increase). Of interest, a 2-day exposure to ACTH enabled cultured ZG cells to respond to  $5 \times 10^{-8}$  M ET-1 by raising their basal release of aldosterone. This finding makes likely the possibility that ACTH sensitized ZG cells to ET-1 via the activation of adenylate cyclase. Quite different results were obtained by Cozza and Gomez-Sanchez (Cozza et al., 1989, 1992; Cozza and Gomez-Sanchez, 1990, 1993). They found that ET-1 concentration-dependently increased basal aldosterone secretion of cultured ZG cells, with minimal and maximal effective concentrations being  $10^{-10}$  M (2-fold rise) and  $10^{-8}$  M (4-fold rise), respectively. Big-ET-1 and ET-3 were less effective than ET-1, whereas ET-2 and STXb were equipotent with ET-1. ET-1 also potentiated maximal ANG-II-stimulated aldosterone secretion ( $10^{-10}$  M and  $10^{-8}$  M ET-1; 70 and 100%, respectively), acting on both early and late steps of steroid synthesis. ET-1 also potentiated the aldosterone response to ACTH, although to a lesser extent than that to ANG-II, but it did not affect the response to  $K^+$ .

c. DOG AND RABBIT ADRENOCORTICAL CELLS. ET-1 infusion (i.v. 30 ng/kg · min) induced a sizeable increase in both PRA and plasma aldosterone concentration (PAC) in the dog (Goetz et al., 1988; Miller et al., 1989, 1993). ET-1 enhanced basal aldosterone but not corticosterone secretion of dispersed rabbit adrenocortical cells. The response was moderate (maximal effective concentration elicited a 26% rise), although ZG cells displayed a very high sensitivity to ET-1 (EC<sub>50</sub>,  $5 \times 10^{-14}$  M).

d. RAT ADRENOCORTICAL CELLS. Consistent evidence indicates that ETs enhance basal aldosterone and corticosterone production by dispersed ZG and ZF/R cells, respectively. However, marked differences were reported with regards to the intensity of the response and the sensitivity to the peptides. The minimal effective concentration of ET-1 ranged from  $10^{-14}$  to  $10^{-10}$  M. The maximal effective concentration ( $10^{-8}$ / $10^{-7}$  M) evoked rises varying from 50% (Woodcock et al., 1990b) to 2- or 3-fold (Mazzocchi et al., 1990a; Belloni et al., 1995, 1996c, 1997) or even to 15- or 30-fold (Hinson et al., 1991a,b). The reasons for these differences are unclear, but they are likely to depend on the experimental conditions. Similar secretagogue effects were also exerted by ET-2, ET-3, and BQ-3020, although these agonists were less effective than ET-1 (Hinson et al., 1991a; Belloni et al., 1997). ET-1 also was found to enhance cholesterol uptake and incorporation into the mitochondrial fraction of adrenocortical cells and to stimulate P450<sub>scc</sub> activity, thereby suggesting that ET-1 acted on the early steps of steroid synthesis in the rat, also (Romero et al., 1996; Pecci et al., 1998). Only one study dealt with the effect of ETs on agonist-stimulated secretion (Mazzocchi et al., 1990a). ET-1 was found to potentiate the aldosterone response of ZG cells to  $10^{-8}$  M ACTH, but not to both ANG-II and  $K^+$ . Minimal and maximal effective concentrations were  $10^{-12}$  and  $10^{-8}$  M, and elicited 40 and 50% rises, respectively. In vivo studies gave rather inconsistent results, possibly attributable to the dose and route of ET-1 administration. Mazzocchi et al. (1990a) did not observe any effect of the bolus i.v. administration of ET-1 (0.5 μg/kg) on PAC within 60 min, whereas Cao and Banks (1990) showed that the i.v. infusion of ET-1 (100 ng/kg · min) elicited a 2- to 3-fold rise. The 7-day s.c. infusion of ET-1 (0.2 μg/kg · h) evoked a clear-cut increase in PAC, coupled with an increase in the systolic blood pressure (BP) and a lowering in PRA (Mazzocchi et al., 1990b). Pecci et al. (1993) reported that ET-1 i.v. infusion exerted a biphasic effect on PAC and cytosolic aldosterone content of ZG cells, the effects being related inversely to the dose (from 50 to 110 ng/kg · min) and directly to the infusion rate (from 50 to 180 μl/min). These investigators also confirmed that ET-1 stimulated both early and late steps of aldosterone synthesis (Fig. 3), but an effect on the conversion of cholesterol to pregnenolone was observed only at the lower dose of the peptide. In contrast with their earlier findings, Mazzocchi et al. (1996) reported that the i.p.

bolus injection of ET-1 dose-dependently (from 0.1 to 10 nmol/rat) raised not only basal, but also ANG-II-enhanced PAC. The in vivo stimulatory effect of ET-1 on aldosterone secretion did not involve any change in the renin-angiotensin-system activity, inasmuch as the angiotensin-converting enzyme inhibitors captopril and enalapril did not affect it (Cao and Banks, 1990; Pecci et al., 1993). Moreover, evidence indicates that ETs inhibit more than stimulate renin release by kidney juxtaglomerular cells (Takagi et al., 1989; Moe et al., 1991; Yamada and Yoshida, 1991; Naess et al., 1993; Galindo-Pacheco et al., 1996; Ritthaler et al., 1996). According to Mazzocchi et al. (1990a,b), ET-1 did not change plasma corticosterone concentration (PBC). However, more recent investigations showed that the bolus s.c. administration of ET-1 (1 nmol/kg) caused a sizeable rise in PBC (Malendowicz et al., 1997c,d). According to these investigators, this effect of ET-1 was possibly related to the stimulating action of this peptide on hypophyseal ACTH release (Calogero et al., 1994; Yasin et al., 1994; Greer et al., 1995; Stojilkovic and Catt, 1996).

e. INTERRENAL CELLS OF LOWER VERTEBRATES. Only frog (*Rana ridibunda*) interrenal cells were studied by an in vitro perfusion system. ET-1 concentration-dependently (from  $10^{-11}$  to  $10^{-8}$  M) acutely raised the basal output of both aldosterone and corticosterone. The responses of the two hormones were similar and achieved their maximum (60% rise) at a ET-1 concentration of  $5 \times 10^{-9}$  M. The prolonged or repeated exposure to ET-1 evoked a rapid desensitization of interrenal cells (Delarue et al., 1990). ET-3 was found to be about 410 times less potent than ET-1 (Cartier et al., 1997). The secretory response to the maximal effective concentration of ET-1 was reversibly blocked by the microfilament-disrupting agent cytochalasin-B, but not by vinblastine and  $\beta$ - $\beta^1$ -iminodinitropropionitrile, which are antimicrotubular and anti-intermediate filament agents (Remy-Jouet et al., 1994). However, the specificity of the effect of cytochalasin-B on ET-1 secretagogue action in frog interrenals seems to be ruled out by the following evidence. Cytochalasin-B also lowered basal steroid release by the frog-interrenal preparations (Remy-Jouet et al., 1994), as well as inhibited in both frogs and humans the stimulating effect of several agonists, including ACTH, ANG-II, serotonin, acetylcholine, and calcitonin gene-related peptide (Netchitaïlo et al., 1985; Feuilloley et al., 1988, 1994; Delarue et al., 1997). The involvement of the cytoskeleton in the basic mechanism underlying steroid synthesis in steroid-secreting tissues is well known (for review, see Feuilloley and Vaudry, 1996), thereby making it likely that cytochalasin-B acted nonspecifically by disrupting the steroidogenic machinery.

2. *Testis*. ET-1 concentration-dependently increased basal testosterone secretion of dispersed rat Leydig cells. The minimal effective concentration was  $10^{-11}$ / $10^{-10}$  M, and the maximal effective concentration ( $10^{-9}$  M) elicited rises ranging from 3- to 5-fold (Conte et al.,

1993; Belloni et al., 1996a; Romanelli et al., 1997). ET-1 exhibited a clear-cut additive stimulatory effect on both hCG- and LH-releasing hormone submaximally stimulated testosterone production (Conte et al., 1993; Romanelli et al., 1997). According to Conte et al. (1993), ET-3 exerted effects similar to those of ET-1 but was much less effective (maximal effective concentration was  $10^{-7}$  M and evoked only a 2-fold rise in basal testosterone output). By using the murine Leydig tumor cell line MA-10, Ergul et al. (1993) showed that ET-1 (from  $10^{-10}$  to  $10^{-6}$  M) enhanced basal progesterone release in a concentration-dependent fashion, the maximal effective concentration evoking about a 6-fold increase.

3. *Ovary*. a. HUMAN OVARY. Using cultures of granulosa-luteinized cells obtained from patients undergoing an IVFET program, Kamada et al. (1995) showed that  $10^{-7}$  M ET-1, ET-3, and IRL-1620 decreased basal and FSH-but not hCG-stimulated progesterone release without affecting estradiol production. Conversely, Mancina et al. (1997) did not observe any effect of ET-1, ET-3, or STXc on either basal estrogen or progesterone release by cultured human granulosa-lutein cells, and Apa et al. (1998) reported an ET-1-induced inhibition of the hCG-stimulated progesterone secretion.

b. CALF OVARY. There is general consensus that ET-1 ( $10^{-8}$ / $10^{-7}$  M) significantly inhibited either basal and LH-stimulated progesterone production by lutein cells (Girsch et al., 1996a; Liebermann et al., 1996; Miyamoto et al., 1997). ET-3 was ineffective, and BQ-610 abolished the inhibitory action of ET-1 (Girsch et al., 1996a); conversely, the effect of ET-1 was potentiated by PGF $2\alpha$ . Investigations with a microdialysis system implanted into the thecal layer of preovulatory follicles maintained in organ culture chambers showed that ET-1 infusion decreased progesterone and androstenedione release and raised estradiol production (Acosta et al., 1998). These investigators also demonstrated that: 1) ET-1 concentration in the theca layer exceeded by about 100-fold that in the blood; 2) LH increased not only the release of progesterone, androstenedione, and estradiol, but also of ET-1; and 3) ET-1 affected PGF $2\alpha$  release by organ-cultured follicles. On these grounds, they suggested that ET-1 may play a key role in a local intermediary/amplifying system of the LH-triggered ovulatory cascade.

c. PIG OVARY. ET-1 concentration-dependently lowered progesterone production by LH- or FSH-stimulated cultured granulosa cells (Iwai et al., 1991; Flores et al., 1992). Progesterone release by luteinized cultured granulosa cells was also decreased by ET-2 and ET-3 ( $5 \times 10^{-8}$  M; Iwai et al., 1991). By using cultures of granulosa cells harvested from small- and medium-sized follicles, a biphasic effect of ET-1 was observed (Kamada et al., 1992, 1993a; Kubota et al., 1994). In short-term (2-h) cultures, ET-1 concentration-dependently enhanced both basal and FSH-stimulated progesterone secretion without affecting estradiol production; conversely, in 48-h cultures,

ET-1 concentration-dependently inhibited FSH-stimulated progesterone secretion without altering the basal one.

d. **RAT OVARY.** Earlier studies reported that both ET-1 and ET-3 (from  $4 \times 10^{-10}$  to  $4 \times 10^{-5}$  M) enhanced the production of progesterone, estradiol, and testosterone by ovaries from immature pregnant mare serum-treated rats, incubated in vitro under static or dynamic (perfusion) conditions (Usuki et al., 1991a, 1992). Usuki et al. (1993) also showed that ET-1 raised basal progesterone, but not estradiol, secretion of cultured granulosa cells. However, the bulk of subsequent investigations clearly demonstrated an evident inhibitory effect of ETs on ovarian steroidogenesis. A marked concentration-dependent inhibitory effect of ET-1 on FSH-stimulated progesterone and estradiol release by cultured granulosa cells from immature or estrogen-primed animals was reported by several groups (Tedeschi et al., 1992, 1994; Otani et al., 1996; Calogero et al., 1998; Usuki et al., 1998). Similar effects were exerted by ET-2 and ET-3, which were equipotent with ET-1 (Tedeschi et al., 1994; Usuki et al., 1998). In contrast, Mancina et al. (1997) and Calogero et al. (1998) showed that ET-3 and STXc were more effective than ET-1 in suppressing basal and stimulated estrogen production, and that their action was unaffected by BQ-123. According to Tedeschi et al. (1992) the maximal effective concentration of ET-1 ( $10^{-9}$  M) not only elicited a 90% inhibition of FSH-stimulated progesterone secretion, but also caused a marked reduction in pregnenolone production and a sizeable increase in the accumulation of progesterone-inactive metabolites (20 $\alpha$ -dihydroprogesterone, pregnanediol, and pregnanolone). Hence, the progesterone antiseoretagogue action of ET-1 may result from the combined inhibition of the early step of progesterone synthesis (i.e., P450scc) and stimulation of progesterone-inactivating enzymes (20 $\alpha$ -HSD and 5 $\alpha$ -reductase).

### *B. Mechanisms of Action and Receptor Subtypes Involved*

1. **Adrenal Cortex.** Several mechanisms appear to be involved in the mediation of ET secretagogue action on adrenal cortex. ETs may act directly on adrenocortical cells by activating several signaling pathways or indirectly by activating mechanisms located outside adrenocortical cells, which in turn may enhance their secretory activity. Obviously, the indirect mechanisms may be operative only in vivo or in experimental models where the integrity of adrenal cortex is preserved.

a. **DIRECT MECHANISMS: SIGNALING CASCADES. i. Stimulation of PLC-dependent cascade and Ca<sup>2+</sup> influx.** Evidence is available that ET-1, like other adrenocortical-cell agonists (see *Section IIB*), exerts its aldosterone secretagogue action by activating PLC, thereby releasing DAG and IP<sub>3</sub>, which leads to the activation of PKC and increases [Ca<sup>2+</sup>]<sub>i</sub> by enhancing both Ca<sup>2+</sup> release from intracellular stores and Ca<sup>2+</sup> influx.

Both ET-1 and ANG-II were found to evoke IP<sub>3</sub> accumulation and to raise [Ca<sup>2+</sup>]<sub>i</sub> in dispersed rat ZG cells. However, ET-1 was three times less potent than ANG-II (Woodcock et al., 1990a,b). Similar results were obtained recently in cultured human ZG cells (Mathieu et al., 1998). Although Rosolowsky and Campbell (1990) did not observe any effect of ET-1 on [Ca<sup>2+</sup>]<sub>i</sub> in cultured bovine ZG cells, the involvement of Ca<sup>2+</sup> in the ZG cell response to ET-1 has been confirmed by several investigators. The Ca<sup>2+</sup>-channel blocker nifedipine inhibited the aldosterone response to ET-1 of both human adrenal quarters (Zeng et al., 1992) and dispersed rabbit ZG cells (Morishita et al., 1989). Moreover, perfusion with Ca<sup>2+</sup>-free medium abolished the secretagogue action of ET-1 on frog interrenal cells (Delarue et al., 1990). According to Cozza and Gomez-Sanchez (1993), the incubation of bovine ZG cell cultures in low Ca<sup>2+</sup> media or in the presence of the Ca<sup>2+</sup>-channel antagonist verapamil suppressed aldosterone response to ET-1, but not the ET-1-evoked potentiation of ANG-II stimulatory effect. In contrast, PKC inhibition by staurosporine and PKC desensitization by ACTH or prolonged phorbol 12-myristate 13-acetate (PMA) exposure did not alter aldosterone response to ET-1 but blocked the potentiating effect of ET-1 on ANG-II secretagogue effect. Cozza and Gomez-Sanchez (1993) concluded that two different signaling cascades mediate the stimulatory action of ET-1 on basal and ANG-II-stimulated aldosterone production by bovine ZG cells. One is Ca<sup>2+</sup> influx-dependent and PKC-independent, and the other is Ca<sup>2+</sup> influx-independent and PKC-dependent. The ET-1-induced activation of PLC and Ca<sup>2+</sup> influx, through both voltage-gated and receptor-operated Ca<sup>2+</sup> channels, has been recently confirmed (Pecci et al., 1998). However, according to these investigators, the PLC activation-dependent DAG and IP<sub>3</sub> production would not be involved in the aldosterone response of rat ZG cells to ET-1, their role remaining unclear and possibly related to the potentiating action of the peptide on agonist-stimulated aldosterone secretion. This hypothesis, although appealing, is not supported by the demonstration that the specific PKC inhibitor Ro-31-8220 lowered by about 75% the ET-1-induced rise in basal aldosterone secretion of dispersed rat ZG cells (Kapas and Hinson, 1996). Cartier et al. (1999) demonstrated that the same effect was exerted by the PLC inhibitor U-73122 in perfused frog adrenal slices. They also showed that ET-1 concentration-dependently enhanced IP<sub>3</sub> production and decreased membrane polyphosphoinositide content in these preparations.

**ii. Stimulation of Tyrosine Kinase Activity.** Both ET-1 and PMA were found to concentration-dependently stimulate TK activity in dispersed rat ZG cells (Kapas and Hinson, 1996), a response abolished by the TK inhibitor tyrphostin-23 and partially reversed by Ro-31-8220. Tyrphostin-23 caused a 30% lowering of the maximum aldosterone response to both ET-1 and PMA. Of interest, ACTH and db-cAMP, which per se did not affect



TK activity in rat ZG cells (Kapas et al., 1995), significantly inhibited TK response to ET-1, ANG-II, and PMA. On these grounds, Kapas and Hinson (1996) concluded that: 1) the TK cascade plays a role in the mediation of the aldosterone secretagogue effects of ETs, its activation involving both PKC-dependent and PKC-independent pathways; and 2) in the rat ZG cells, a notable cross-talk occurs between TK-linked receptors and receptors coupled with the PLC/PKC and adenylate cyclase/PKA signaling pathways.

**iii. Stimulation of Adenylate Cyclase-Dependent Cascade.** Cartier et al. (1999) proposed that such a mechanism plays a role in amphibia interrenal cells. The ET-1-induced stimulation of aldosterone and corticosterone secretion of frog interrenal cells was partially blocked by the PKA-selective inhibitor H-89. Moreover, ET-1 caused a sizeable dose-related increase in cAMP release by frog preparations. The simultaneous exposure to U-73122 and H-89 completely suppressed the ET-1 effect, thereby suggesting the involvement of both PLC- and adenylate cyclase-dependent pathways.

**iv. Stimulation of Cyclooxygenase-Dependent Cascade.** ET-1 was found to markedly enhance the release of PGE<sub>2</sub> and 6-keto-PGF<sub>1</sub> $\alpha$ , the stable PGE<sub>2</sub> metabolite, by frog interrenal slices. The rise in the PGE<sub>2</sub> biosynthesis occurred 10 min earlier than the peak of the corticosteroid response to ET-1 (Delarue et al., 1990). The possibility that the CO-dependent pathway plays a role in the response of frog interrenal gland to ETs is also supported by the demonstration that the secretory response was suppressed by the CO inhibitor indomethacin.

**v. Stimulation of Ouabain-Sensitive Na<sup>+</sup>/K<sup>+</sup>-ATPase.** ET-1 infusion, in addition to increasing BP and adrenal aldosterone content, also caused a clear-cut activation of microsomal Na<sup>+</sup>/K<sup>+</sup>-ATPase in rat ZG cells (Pecci et al., 1994, 1998). Similar results were obtained by infusing STXb. According to these investigators, this effect may play a role in mediating the ET-1-induced potentiation of the aldosterone response to ANG-II. ANG-II has no direct effect on adrenal Na<sup>+</sup>/K<sup>+</sup>-ATPase (Meuli and Müller, 1982), but it activates Na<sup>+</sup>-H<sup>+</sup> exchange, resulting in a marked increase in the intracellular (cytosolic) sodium concentration ([Na<sup>+</sup>]<sub>i</sub>; Conlin et al., 1990, 1991): ET-1-induced stimulation of Na<sup>+</sup>/K<sup>+</sup>-ATPase activity could decrease [Na<sup>+</sup>]<sub>i</sub>, thereby enhancing the Na<sup>+</sup> gradient across the plasma membrane and allowing faster intracellular alkalinization by ANG-II. In this connection, it seems of interest to recall that ouabain and ouabain-like compound (OLC) bind to and inhibit cell-surface Na<sup>+</sup>/K<sup>+</sup>-ATPase, thereby regulating Na<sup>+</sup> and K<sup>+</sup> fluxes, and many lines of evidence indicate that mammalian ZG cells are able to release an endogenous OLC (for review, see Hinson et al., 1995; Foster et al., 1998), although conflicting reports also exist (Rossi et al., 1995c). The effects of ouabain on the secretory activity of ZG cells are controversial. Some investigators

reported that ouabain counteracted the aldosterone response to ANG-II (Elliot et al., 1986; Antonipillai et al., 1996), whereas others showed that ouabain enhanced basal aldosterone secretion (Szalay, 1993, 1998; Tamura et al., 1996; Yamada et al., 1996). ANG-II, via the AT<sub>2</sub>-receptor subtype, was found to increase OLC release by bovine adrenocortical cell cultures (Shah et al., 1998). The inhibition of Na<sup>+</sup>/K<sup>+</sup>-ATPase by ouabain was found to be associated with a moderate enhancement of aldosterone production by dispersed rat ZG cells. Ouabain and ET-1 acted synergistically in this cell preparation (Pecci et al., 1998). Hence, the modulation of Na<sup>+</sup>/K<sup>+</sup>-ATPase through autocrine/paracrine mechanisms, involving ETs and OLC, might take part in the fine-tuning of ZG aldosterone secretion.

**b. DIRECT MECHANISMS: RECEPTOR SUBTYPES.** Naruse et al. (1994b) reported that BQ-123 slightly but significantly suppressed the aldosterone secretagogue effect of ET-1 in cultured bovine ZG cells, thereby suggesting the involvement of the ETA receptor. However, the bulk of investigations strongly suggests that the ETB receptor is the main subtype mediating both the mineralocorticoid and glucocorticoid response of bovine and rat adrenocortical cells to ETs. As reviewed above, ET-3 and STXb were found to be equipotent with ET-1 in evoking the aldosterone response, and BQ-123 was ineffective in blocking ET-1-induced increase in Na<sup>+</sup>/K<sup>+</sup>-ATPase activity and cholesterol uptake by ZG cells (Pecci et al., 1994, 1998; Romero et al., 1996). Further insight was gained by the use of selective agonists and antagonists of ETB receptors. Belloni et al. (1996c, 1997) showed that BQ-788, but not BQ-123, suppressed either the aldosterone or the corticosterone response of dispersed rat ZG and ZF/R cells to ET-1, ET-3, and BQ-3020. Consistent with these findings, Mazzocchi et al. (1996) reported that BQ-788, but not BQ-123, abolished the *in vivo* ET-1-induced potentiation of PAC response to ANG-II. According to Belloni et al. (1996a, 1997), minimal and maximal *in vitro* concentrations of BQ-788 were 10<sup>-9</sup> and 10<sup>-7</sup> M, respectively. However, 10<sup>-7</sup> M BQ-788, either alone or with equimolar concentrations of BQ-123, although abrogating the glucocorticoid response of ZF/R cells, only decreased the aldosterone response to ET-1 of ZG cells by about 70%. In light of their findings, these investigators advanced the hypothesis that in the rat, a third subtype of ET receptors (see Section IIB) or an alternatively spliced receptor (Miyamoto et al., 1996) may also be involved in the aldosterone secretory response of ZG cells to ETs. Kapas et al. (1996) confirmed that the ETB antagonists, although completely abolishing the secretory response of rat ZF/R cells to ET-1, only partially reversed that of ZG cells. In contrast, they observed that BQ-123 exerted a clear-cut inhibitory action and that a complete reversal of ET-1-stimulated ZG secretion could be obtained by using equimolar (10<sup>-8</sup> M) concentrations of RES-701-1 and BQ-123. Accordingly, they suggested that in the rat, ZG ETA receptors are

functionally more important than ETB receptors in mediating aldosterone response to ETs. Further studies, possibly using selective ligands of the various subtypes of ETA receptors (e.g.,  $ETA_1$  and  $ETA_2$ ; see Section IIB), are required to explain the above-described discrepancies. Also, differences in the strain and substrain of rats used conceivably may account for the different results obtained by Belloni et al. (1996a, 1997) and Kapas et al. (1996). The subtype of receptors mediating the adrenocortical secretagogue action of ETs does vary according to the species. In this connection, it is worth mentioning that recent findings showed that BQ-123 blocked the ET-1-induced DAG production in rat ZG cells, thereby suggesting that ETA receptors mediate ET-1-evoked PLC/PKC activation (Pecci et al., 1998). However, according to these investigators, the PKC-dependent cascade is not involved in the basal aldosterone response to ET-1. Rossi et al. (1997b) provided convincing evidence that both ETA and ETB receptors were involved in the mediation of aldosterone and cortisol responses to ET-1 of human adrenocortical cells, the two receptor subtypes being about equipotent. A complete blockade of  $10^{-8}$  M ET-1-stimulated secretion of both ZG and ZF/R cells was obtained only by combining equimolar concentrations ( $10^{-7}$  M) of BQ-123 and BQ-788. Cartier et al. (1997) reported that the secretory response of frog adrenocortical cells to both ET-1 and ET-3 was blocked by either  $10^{-5}$  M Ro-47-0203 (bosentan), a mixed ETA/ETB antagonist, or  $10^{-7}$  M BQ-485, a selective ETA receptor antagonist. The ETB-receptor antagonist IRL-1038 ( $10^{-6}$  M) was ineffective, and the ETB-receptor agonist IRL-1620 ( $10^{-6}$  M) did not mimic the stimulatory effect of ETs. Although the selectivity and effectiveness of IRL-1038 is questionable (Urade et al., 1994), these findings strongly suggest that in the frog adrenocortical cells, the corticosteroid response to ETs is exclusively mediated by the ETA receptors. In this connection, we wish to recall that only in this lower-vertebrate species does the mechanism of the adrenocortical secretagogue action of ETs seem to involve the simultaneous activation of the PLC- and adenylate cyclase-dependent signaling pathways.

c. INDIRECT MECHANISMS. **i. Stimulation of Catecholamine Release by Adrenal Medulla.** The adrenomedullary catecholamines, epinephrine and norepinephrine, are known to exert a paracrine-stimulatory control of the cortex function in both mammalian and lower vertebrate adrenals (for review, see Nussdorfer, 1996; Ehrhart-Bornstein et al., 1998; Mazzocchi et al., 1998a). This effect of catecholamines is mediated by both  $\alpha$ - and  $\beta$ -adrenoceptors located on adrenocortical cells, and impacts both mineralocorticoid and glucocorticoid secretion. Several studies have provided evidence that many regulatory peptides contained in adrenal glands may exert their adrenocortical secretagogue effect through such an indirect mechanism. At present, this group of peptides includes interleukin (IL)-1, Substance P, neu-

ropeptide Y, vasoactive intestinal polypeptide, pituitary adenylate cyclase-activating polypeptide, adrenomedullin (for review, see Nussdorfer, 1996, 1997b; Nussdorfer and Malendowicz, 1998a,b), and cerebellin (Mazzocchi et al., 1999). The following evidence suggests that ETs may act in vivo through this mechanism. ET-1 markedly increased catecholamine release by cultured bovine adrenomedullary cells through a mechanism dependent on extracellular  $Ca^{2+}$  (Boarder and Marriott, 1989, 1991; Ohara-Imaizumi and Kumakura, 1991). The ET-1 infusion into the left adrenolumbar artery of anesthetized dogs evoked a dose-dependent increase in catecholamine output into the adrenal venous effluent, an effect blocked by nifedipine, and therefore conceivably mediated by the activation of voltage-gated  $Ca^{2+}$  channels (Yamaguchi, 1993, 1995, 1997). ET-1, and to a lesser extent ET-3 and BQ-3020, elicited the release of catecholamine by rat adrenal medulla fragments (Belloni et al., 1997), and ET-1, ET-2, and ET-3 raised phosphoinositide turnover in rat adrenal chromaffin cells (Garrido and Israel, 1997). Hence, ETs appear to stimulate adrenomedullary cell secretion through the activation of PLC-dependent cascade and  $Ca^{2+}$  influx. Recently, Rebuffat et al. (1999) obtained findings directly demonstrating that this indirect mechanism may concur with the direct ones in the aldosterone secretagogue effect of ET-1. The aldosterone response of adrenal slices containing medullary chromaffin cells to ET-1 was more intense than that of dispersed ZG cells (the minimal effective concentrations were  $10^{-11}$  and  $10^{-10}$  M, and the maximal effective concentration was  $10^{-8}$  M, eliciting 4.8- and 3.4-fold rises, respectively). The  $\beta$ -adrenoceptor antagonist *l*-alprenolol partially reversed (by about 55%) the aldosterone secretagogue effect of the maximal effective concentration of ET-1 on adrenal slices, but not on dispersed ZG cells. By the use of selective antagonists, it has been reported that the main subtype of ET receptors mediating the catecholamine secretagogue action is ETA in the dog and rat (Garrido and Israel, 1997; Yamaguchi, 1997) and ETB in the calf (Houchi et al., 1998). Belloni et al. (1997) showed that both ETA and ETB receptors mediated the stimulating action of ETs on rat adrenal medulla, although ETA seemed to play a major role. Rebuffat et al. (1999) not only confirmed these last findings, but additionally showed that both BQ-788 and BQ-123 counteracted the aldosterone secretagogue effect of ET-1 on adrenal slices and abolished it when administered together at a concentration of  $10^{-7}$  M.

**ii. Modulation of the Release of Intramedullary Arginine Vasopressin (AVP) and ANP.** AVP is a potent stimulator of aldosterone secretion, and proof is available that the adrenal medulla contains and releases sizeable amounts of AVP-LI, which may affect the cortex in a paracrine manner (for review, see Nussdorfer, 1996). Findings indicate that ET-1 and ET-3, either systemically or centrally injected, stimulate AVP secre-

tion by the hypothalamo-neurohypophyseal axis of dogs and rats (Goetz et al., 1988; Shichiri et al., 1989; Nakamoto et al., 1991; Ritz et al., 1992; Wall and Ferguson, 1992; Yamamoto et al., 1992; Yasin et al., 1994). ANP belongs to a family of natriuretic hormones exerting a potent inhibitory action on the aldosterone secretion of ZG cells; they are contained in the adrenal medulla, and evidence suggests that they could exert a paracrine suppression of ZG function, especially when aldosterone production is enhanced (for review, see Nussdorfer, 1996). There is evidence that ETs stimulate ANP synthesis and release by rat atrial cardiocytes through the ETA receptor subtype (Fukuda et al., 1989; Gardner et al., 1991; Uusimaa et al., 1992; Irons et al., 1993; Thibault et al., 1994). Macchi et al. (1999) recently reported that ET-1 concentration-dependently raised AVP-LI release by rat adrenal medulla, with minimal and maximal effective concentrations being  $10^{-9}$  M (50% increase) and  $10^{-7}$  M (2.7-fold increase);  $10^{-7}/10^{-8}$  M ET-1 also evoked a moderate rise in ANP-LI release. The effects of ET-1 on both AVP and ANP release were mediated by the ETA receptors, because they were annulled by BQ-123, but not by BQ-788. It also was demonstrated that a selective antagonist of V1-AVP receptors exerted a significant inhibitory effect on the ET-1 ( $10^{-7}$  M)-induced aldosterone release by rat adrenal slices containing medullary chromaffin cells; conversely, a selective ANP-receptor antagonist was ineffective. These findings are compatible with the possibility that the ET-enhanced release by medullary chromaffin cells of AVP, but not of ANP, may play a role in the in vivo aldosterone secretagogue effect of ETs, at least in the rat (Macchi et al., 1999).

**iii. Regulation of Adrenal Blood Flow.** Compelling evidence indicates that a very close direct correlation between blood flow rate and corticosteroid secretion of the adrenal glands exists, at least in the rat (for review, see Vinson and Hinson, 1992). The mechanisms underlying this phenomenon may involve: 1) washout of secretory products; 2) enhancement in the rate of presentation of agonists; 3) rise in oxygen and substrate supply; and 4) flow-dependent changes in the biosynthesis of endothelium-dependent factors. Moreover, medullary adrenal veins, especially the central one, are able to contract, thereby regulating hormonal discharge (for review, see Bassett and West, 1997). Hence, any regulatory molecule that is able to modulate adrenal blood flow may indirectly influence the steroid secretion of the gland. By the use of the model of in situ perfusion of the rat left adrenal gland, it has been shown that the bolus injection into the perfusate of 10 pmol of ET-1 provoked an abrupt fall (50%) in the flow rate of the perfusion medium, which rapidly subsided in about 20 min (Cameron et al., 1994). Thus, it is possible that local intraglandular mechanisms are operative that antagonize the characteristically long-acting and difficult-to-washout vasoconstrictor effect of ET-1. ETs are well known to

elicit the release by the endothelial lining of NO, which provokes acute vasodilation (for review, see Moncada et al., 1991; Rubanyi and Polokoff, 1994; Cooke and Tsao, 1996). It has been suggested that by activating guanylate cyclase, NO may have a major role in the termination of ET signaling (Goligorsky et al., 1994). NO is released in the adrenal gland and surely may play a role in the local regulation of blood flow (Breslow, 1992; Torres et al., 1994), and, consequently, of steroid release. Convincing evidence of this latter action of NO was provided by Cameron and Hinson (1993). Using the in situ perfusion model, they demonstrated that the NO synthase (NOS) substrate L-arginine dose-dependently increased perfusion-medium flow rate and steroid output, whereas the NOS inhibitor  $N^G$ -nitro-L-arginine (L-NAME) decreased these two parameters even in the presence of L-arginine. Hinson et al. (1996a,b) confirmed these findings, and additionally showed that the injection of sodium nitroprusside, an endothelium-independent activator of guanylate cyclase, markedly raised the flow rate of the perfusion medium, a finding in keeping with the view that NO enhances adrenal blood flow by activating cGMP production. According to Gellai et al. (1996), in the rat blood vessels, the predominant role of endogenous ETs is an ETB receptor-mediated vasodilation, ETA receptors playing a negligible role in the control of vascular tone. However, recent investigations indicate that both ETA and ETB receptors play a role in the regulation of vascular tone and blood flow in the rat adrenals. Mazzocchi et al. (1998b) showed that ET-1 decreased the flow rate of the perfusion medium in the in situ perfused rat adrenal, and this effect was reversed by BQ-123 and enhanced by BQ-788. ET-3 and BQ-3020 increased the perfusate flow rate, and their effects were annulled by BQ-788. BQ-123 magnified the effect of ET-3 but not that of BQ-3020. The ETA receptor-mediated lowering and the ETB-mediated rise in the perfusate flow rate were abolished by the PKC inhibitor Ro-31-8220 and L-NAME, respectively. Mazzocchi and associates (1998b) concluded that ETs can regulate intra-adrenal vascular resistances via both PKC-coupled ETA and NOS-coupled ETB receptors, the activation of which evokes vasoconstriction and vasodilation, respectively. The discrepancies of these findings with those of Gellai et al. (1996) may be easily reconciled by considering that in the in situ perfusion model, exogenous ETs are delivered to the gland periphery by adrenal arteries, and by taking into account the different distribution of ETA and ETB receptors in the rat adrenals (see *Section IVD*). The "strategic" location of the ETA receptors in the subcapsular region of adrenals may explain why the first effect of ETs contained in the perfusate is the constriction of pericapsular and subcapsular arterioles, with the ensuing drop in the flow rate of perfusate. This cannot be efficiently counteracted by the subsequent activation of the ETB receptors mainly located in the endothelial lining of the intra-adrenal cap-

illary network. Another important target of ETs could be the central adrenomedullary vein, the contraction of which conceivably causes a further decrease in the rate of perfusate recovery. This last possibility is supported by the demonstration that, in calves and pigs, ETs, via ETA receptors, induced the contraction of adrenomedullary veins (Lønning and Helle, 1995; Lønning et al., 1997; Bjerckås et al., 1998). According to Mazzocchi et al. (1998b), neither BQ-123 and BQ-788 nor PKC and NOS antagonists were able to affect intra-adrenal vascular resistances, thereby making it difficult to conceive that endogenous, locally released ETs play a relevant role in the physiological regulation of adrenal blood flow (and steroid release). This contention appears to be at variance with the findings reported by Hinson et al. (1996b). They observed that the injection of BQ-123 and L-NAME alone evoked a 20% increase and a 60% decrease, respectively, in the basal flow rate of perfusate, and suggested that endogenous ETs and NO exert a tonic vasoconstrictor and vasodilatory action on rat adrenal vasculature, respectively. According to Cameron et al. (1994), a close direct correlation exists between the flow rate of the perfusion medium and the concentration of ET-1-LI in the perfusate effluent in the in situ perfused rat adrenal (see Section VIIA). Hinson et al. (1996b) used a perfusion rate three times higher than that used by Mazzocchi et al. (1998b). Hence, it is likely that the tonic activation of adrenal vascular ET receptors by locally released ETs has to be considered as an artifact caused by the exceedingly high, nonphysiological rate of adrenal perfusion.

2. *Testis*. As surveyed in Section IV, all investigators agree that Leydig cells are exclusively provided with the ETA receptor subtype. Accordingly, the testosterone secretory response to ET-1 of dispersed rat Leydig cells was concentration-dependently impaired by BQ-123, a complete blockade being attained at concentrations higher than  $10^{-7}$  M. Conversely, BQ-788 was ineffective, thereby ruling out an involvement of the ETB receptors (Belloni et al., 1996a; Romanelli et al., 1997). As in the case of the adrenal cortex, the mechanism underlying the secretagogue action of ETs on Leydig cells may be direct and indirect. However, these last possibilities are only hypothetical at present.

a. DIRECT MECHANISMS: SIGNALING CASCADES. **i. Stimulation of PLC-dependent cascade and  $Ca^{2+}$  influx.** ET-1 was found to evoke contraction of the rat peritubular myoid cells (Filippini et al., 1993; Tripiciano et al., 1996, 1997), and to inhibit FSH-stimulated cAMP and estradiol production by Sertoli cells through a PKC-dependent pathway (Sharma et al., 1994). However, the direct demonstration that ETs stimulate Leydig-cell secretion through this signaling mechanism is lacking. The stimulatory action of ET-1 on rat Leydig cells was blocked by nifedipine, thereby suggesting the involvement of a  $Ca^{2+}$ -influx rise (Conte et al., 1993). However, according to Tomic et al. (1995), rat Leydig cells do not express voltage-gated  $Ca^{2+}$  channels, inasmuch as their

$[Ca^{2+}]_i$  was unaffected by either  $K^+$  or the L-type  $Ca^{2+}$  channel agonist BAYK-8644. These investigators showed that ET-1 caused a rapid and transient rise in  $[Ca^{2+}]_i$ , which was not associated with a subsequent sustained plateau phase and was not affected by the removal of  $Ca^{2+}$  from the incubation medium. The amplitude of  $Ca^{2+}$  response did not depend on the ET-1 concentration, but the peptide concentration-dependently (from  $10^{-8}$  to  $2 \times 10^{-8}$  M) raised the number of responsive cells to a maximum of 30%. As suggested for Sertoli cells (Sharma et al., 1994), ET-1-induced  $Ca^{2+}$  transient may be dependent on  $Ca^{2+}$  mobilization from intracellular stores through the activation of the PLC-dependent signaling cascade and  $IP_3$  production.

ii. *Stimulation of Cyclooxygenase-Dependent Cascade.* Conte et al. (1993) reported that ET-1 (from  $10^{-9}$  to  $10^{-10}$  M) increased PGE2 release by rat Leydig cells, but denied the PG involvement in the testosterone secretory response to ETs because indomethacin did not affect it. Subsequent studies of the same group (Romanelli et al., 1997) confirmed that ET-1 stimulated basal and potentiated LH-releasing hormone-enhanced PGE2 release (2-fold and 84% rise, respectively), and additionally showed that the PLA2 inhibitor quinacrine (but not the LO blocker nordihydroguaiaretic acid) abolished either testosterone or PGE2 response of Leydig cells to ET-1. These findings strongly suggest that ET-1 exerts its testosterone secretagogue effect by activating the CO pathway of arachidonic acid metabolism.

b. DIRECT MECHANISMS: RECEPTOR SUBTYPES. Belloni et al. (1996a) demonstrated that BQ-123, but not BQ-788, abolished ET-1-induced rise in testosterone production by dispersed rat Leydig cells. By indicating the involvement of the ETA receptors, this observation accords well with their exclusive expression in Leydig cells (see Section IVA).

c. INDIRECT MECHANISMS. The intratesticular injection of ET-1 dose-dependently decreased testicular blood flow in the rat, as monitored using laser Doppler flowmetry. The minimal and maximal effective doses were 0.1 and 100 ng, and elicited 7 and 30% lowering, respectively. The duration of the response was also dose-dependent, lasting 60 min after the injection of 100 ng of ET-1. Blood-flow response to ET-1 was blocked by BQ-123, thereby indicating the involvement of the ETA receptor subtype (Collin et al., 1996). These results raise the interesting possibility that ETs may modulate testosterone release through changes in the blood flow rate of the testis.

3. *Ovary*. a. MECHANISMS OF ACTION. **i. Stimulation of PLC-Dependent Cascade.** ET-1 concentration-dependently ( $10^{-9}$ – $10^{-5}$  M) stimulated phosphoinositide hydrolysis and evoked a transient  $[Ca^{2+}]_i$  increase in the cultured granulosa cells of humans (Kamada et al., 1995) and pigs (Kamada et al., 1992; Flores et al., 1992; Kubota et al., 1994). ET-1 concentration-dependently raised the percentage of  $Ca^{2+}$  responding cells, which

reached 93% at a peptide concentration of  $10^{-5}$  M (Flores et al., 1992); this figure was reduced to about 36% by pretreating cultures with the PLC inhibitor U-73122 (Flores et al., 1998). This last finding, coupled with the demonstration that the  $10^{-7}$  M ET-1-evoked rise in  $[Ca^{2+}]_i$  was not affected by either the voltage-gated  $Ca^{2+}$ -channel blocker nifedipine or the extracellular chelating agent EDTA (Kamada et al., 1992; Kubota et al., 1994), strongly suggests that ETs stimulate PLC-dependent  $Ca^{2+}$  release from intracellular stores. The PLC-induced activation of PKC may also play a role, inasmuch as PMA mimicked ET-1-evoked inhibition of FSH-stimulated progesterone release by cultured swine granulosa cells (Flores et al., 1992). Of interest, ET-1 did not alter  $[Ca^{2+}]_i$  in LH-responsive, dispersed chicken granulosa cells (Sahin-Toth et al., 1990).

**ii. Modulation of Adenylate Cyclase-Dependent Cascade.** Conflicting results on this issue are available. Usuki et al. (1991a, 1992) reported that ET-1 and ET-3, at concentrations over  $10^{-7}$  M, raised cAMP accumulation (along with steroid production) in rat ovaries. Kamada et al. (1992) did not observe any effect of ET-1 on basal and FSH-stimulated cAMP release by cultured pig granulosa cells. In contrast, ETs were found to inhibit LH- or FSH-stimulated cAMP production by cultured pig and rat granulosa cells (Iwai et al., 1991; Flores et al., 1992; Tedeschi et al., 1994; Calogero et al., 1998). Tedeschi et al. (1994) showed that ET-1 also was able to suppress progesterone release evoked by both forskolin and dbcAMP, thereby implying that the peptide may interfere not only with cAMP generation, but also with the PKA-dependent cascade.

**iii. Stimulation of Cyclooxygenase-Dependent Cascade.** Evidence has been provided that LH and ET-1 enhanced  $PGF2\alpha$  release by bovine follicles in organ culture, thereby suggesting the activation of the CO pathway of arachidonic acid metabolism (Acosta et al., 1998). However, Calogero et al. (1998) did not observe any effect of the CO inhibitor indomethacin on rat granulosa cell secretory response to ETs. Nonetheless, a close link appears to exist between  $PGF2\alpha$  and ET-1. In fact,  $PGF2\alpha$  was found to increase ET-1 mRNA in the calf lutein and endothelial cells at day 10 of the cycle (Girsch et al., 1996b) and ET-1 release by corpus luteum slices (Girsch et al., 1996a; Miyamoto et al., 1997).  $PGF2\alpha$  per se did not affect progesterone secretion of dispersed purified bovine lutein cells; however, it inhibited the progesterone secretory response of corpus luteum slices (Girsch et al., 1995). The possibility that the antisecretagogue effect of  $PGF2\alpha$  on corpus luteum slices may be mediated by the local release of ETs, is supported by the demonstration that it was blocked by the ETA receptor antagonist BQ-610 (Girsch et al., 1996a).

**b. RECEPTOR SUBTYPES.** The currently available evidence suggests that ETA receptors mediate the inhibitory effect of ETs on ovarian steroid secretion in humans and calves. Kamada et al. (1995) reported that the effec-

tiveness of ET-3 and IRL-1620 in suppressing progesterone secretion of human granulosa cells was only 16 and 3%, respectively, of that of ET-1, and that the antisecretagogue effect of ET-1 was abolished by BQ-123. Girsch et al. (1996a) showed that ET-1, but not ET-3, inhibited steroid secretion of the bovine corpus luteum, ET-1 effect being suppressed by the ETA receptor antagonist BQ-616. Opposite results were obtained in the rat ovary by Calogero et al. (1998). They reported that ET-3 and STXc were more effective than ET-1 in inhibiting basal and FSH-stimulated estrogen production by cultured granulosa cells; BQ-123 did not influence the ET inhibitory action, thereby suggesting the involvement of ETB receptors.

**4. Other Possible Mechanisms.** We wish now to briefly mention other possible mechanisms by which ETs could influence the secretory activity of steroid-secreting tissues, among which the most probable appear to be that mediated by NOS-coupled ETB receptors, the presence of which appears well demonstrated in the rat adrenal cortex.

**a. STIMULATION OF NITRIC OXIDE GENERATION.** The existence of NOS-coupled ETB receptors involved in the ET-induced regulation of adrenal blood flow has been reviewed above. Findings now have been accumulated that NO can directly affect the secretory activity of steroid-secreting cells, but the unequivocal demonstration that NO generation may interfere with the direct effect of ETs on their secretion is still lacking.

**i. Adrenal Gland.** NOS activity was found in rat and bovine adrenal cytosol, where NO stimulates a soluble guanylate cyclase (Palacios et al., 1989; Bredt et al., 1990). However, in vivo and in vitro studies gave conflicting results as to the role of NO in adrenal steroidogenesis regulation. A possible stimulating effect of NO has been suggested in light of evidence indicating that L-NAME lowered corticosterone secretion by isolated perfused rat adrenals (Cameron and Hinson, 1993), and that another NOS inhibitor prevented ACTH-stimulated aldosterone production by isolated rat adrenals (Nakayama et al., 1996). In contrast, the in vivo administration of L-NAME dose-dependently raised PBC without affecting plasma ACTH level (Adams et al., 1991), and NO was reported to inhibit ANG-II- and ACTH-stimulated aldosterone production by rat and human ZG cells (Natarajan et al., 1997). More recent studies confirmed the inhibitory effect of NO. Different NO donors decreased either basal or dbcAMP and 22(R)OH-cholesterol-enhanced corticosterone secretion of dispersed rat ZF cells, but not corticosterone production elicited by the addition of pregnenolone; NOS inhibitors, on the contrary, raised corticosterone production (Cymeryng et al., 1998). The suppressive effect of NO on ANG-II-stimulated aldosterone production by cultured bovine ZG cells was recently reported to occur through a cGMP-independent inhibition of P450<sub>scc</sub> (Hanke et al., 1998). Taken

together, these observations strongly suggest that NO impairs early steps of adrenocortical steroid synthesis.

**ii. Testis and Ovary.** A direct inhibitory action of NO on sex-hormone production has been reported by several investigations. In vivo, L-NAME raised testosterone blood concentration (Adams et al., 1991), and in vitro, it increased both basal and hCG-stimulated testosterone production by Leydig cells (Welch et al., 1995); accordingly, NO inhibited hCG-stimulated steroidogenesis of dispersed rat Leydig cells (Del Punta et al., 1996). NO was found to impair the secretory activity of cultured rat granulosa cells (Dave et al., 1997) and human granulosa-lutein cells (Van Voorhis et al., 1994). NOS gene expression was detected in mid- and early-phase human corpus luteum, and NO donors elicited a clear-cut inhibitory action on steroid secretion of cultured lutein cells (Vega et al., 1998).

**b. STIMULATION OF PHOSPHOLIPASE D-DEPENDENT CASCADE.** It is recognized that PLD is involved in an alternative pathway, leading to PKC activation and arachidonic acid production (for review, see Exton, 1997). PLD hydrolyzed phosphatidylcholine to phosphatidic acid, which may be dephosphorylated to yield DAG; DAG either activates PKC or is transformed into arachidonic acid by diglyceride lipase (Fig. 4). There is evidence that ET-1 activates PLD in ventricular myocytes, in myometrium, and in astrocytes (Desagher et al., 1997; Clerk and Sugden, 1997; Naze et al., 1997; Servitja et al., 1998), and the possibility that this occurs also in steroidogenic tissues waits to be explored.

**c. STIMULATION OF LOCAL RELEASE OF CYTOKINES.** An increasing mass of findings indicates that locally synthesized and released inflammatory cytokines may variously affect the secretory activity of steroid-secreting tissues, and especially of adrenal cortex (for review, see Nussdorfer and Mazzocchi, 1998). Among these cytokines, a prominent role is played by ILs and tumor necrosis factor  $\alpha$ , which enhance and lower steroid secretion, respectively. Evidence is available now that ET-1 enhances the synthesis and release of IL-6 in the endothelial cells of rat and humans (Xin et al., 1995; Stankova et al., 1996) and bone marrow-derived stromal cells of the rat (Agui et al., 1994), as well as of tumor necrosis factor  $\alpha$  in mouse cultured macrophages (Rutten and Thiernemann, 1997). Hence, the issue that this could also occur in adrenal and other steroid-secreting tissues awaits to be addressed.

### C. Summary

There is now general consensus that ETs stimulate both aldosterone and glucocorticoid secretion by mammalian and amphibian adrenals, probably acting on early and late steps of steroid synthesis. In mammals, the ET secretagogue effect appears to be mediated by both ETA and ETB receptors and to occur through direct and indirect mechanisms. ET binding to its receptors located on adrenocortical cells activates multiple signal-

ing mechanisms, including PLC/PKC cascade, opening of  $\text{Ca}^{2+}$  channels, CO/PG cascade, and stimulation of TK and  $\text{Na}^+/\text{K}^+$ -ATPase. In the amphibia, ETs act exclusively through ETA receptors, and the transduction mechanisms seems also to involve the activation of adenylate cyclase/PKA cascade. Under in vivo conditions or when the integrity of adrenal tissue is preserved, ETs affect the secretory activity of the cortex by eliciting the release by medullary chromaffin cells of catecholamines and AVP, which in turn stimulate steroid secretion in a paracrine manner, and/or by modulating intra-adrenal blood flow. This last effect seems to be mediated by both PKC-coupled ETA- and NOS-coupled ETB-receptors, which raise and lower intraglandular vascular resistances, respectively. A tentative schematic representation of the main autocrine/paracrine feedback mechanisms involved in the ET-mediated control of adrenocortical secretion is depicted in Fig. 5. The effects of ETs on the endocrine gonads have been far less investigated. The bulk of evidence indicates that ETs enhance testosterone secretion by testis Leydig cells, exclusively acting through ETA receptors, the transduction mechanism of which may involve activation of PLC/PKC and CO/PG cascades and opening of  $\text{Ca}^{2+}$  channels. In contrast, ETs strongly inhibit agonist-stimulated estrogen and progesterone secretion by ovarian follicles and corpora lutea, and to a lesser extent, the basal secretion, also. The inhibitory effect appears to be mainly mediated by ETA receptors, although in the rat, the exclusive ETB receptor involvement has been recently proposed to occur through the inhibition of the adenylate cyclase/PKA cascade. Other possible signaling mechanisms include the activation of PLC/PKC and CO/PG cascades, which could negatively interact with adenylate cyclase.

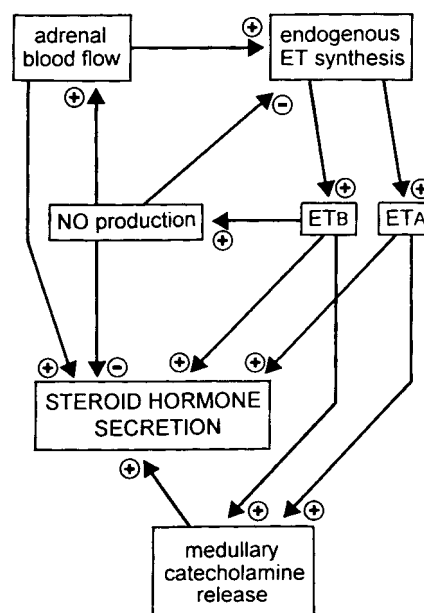


FIG. 5. Main feedback mechanisms currently thought to be involved in the ET-mediated autocrine/paracrine tuning of adrenocortical secretion.

## VI. Effects of Endothelins on the Growth of Steroid-Secreting Cells

### A. Cell Hypertrophy and Proliferation

1. *Adrenal Cortex.* a. ADULT AND JUVENILE ADRENALS. **i. Cell Hypertrophy and Steroidogenic Capacity.** The effects of the prolonged administration of ET-1 on the adrenal cortex of adult rats were studied by Mazzocchi et al. (1990b). Male rats were s.c. infused for 7 days with ET-1 (0.2  $\mu\text{g}/\text{kg} \cdot \text{h}$ ) by Alzet osmotic pumps. In addition to enhancing BP and PAC (see Section VA), the treatment caused a marked hypertrophy of the ZG and its cells, as evaluated by morphometry. The 35% increase in ZG cell volume was caused by increases in the volume of the mitochondrial compartment and SER, the organelles where the enzymes of steroid synthesis are located (see Section IIB). Accordingly, dispersed hypertrophic ZG cells evidenced a significantly increased basal and maximally agonist-stimulated secretory activity (2-fold and 40%, respectively). ZF/R was apparently unaffected. Mazzocchi and associates (1990b) concluded that ET-1 evoked a moderate but significant enhancement in the growth of ZG cells and expression of their steroidogenic enzymatic machinery. However, further investigations casted doubts on the possibility that endogenous ETs play a physiological role in the normal maintenance of the ZG growth in rats because the prolonged treatment with high doses of the ET-receptor antagonists BQ-123 or BQ-788 did not alter tissue status (Malendowicz et al., 1998).

**ii. Cell Proliferation.** Earlier investigations (Cozza and Gomez-Sanchez, 1990) demonstrated that ET-1 concentration-dependently decreased [ $^3\text{H}$ ]thymidine incorporation in cultured bovine ZG cells. Minimal and maximal effective concentrations were  $10^{-10}$  and  $10^{-8}$  M and induced 23 and 57% decreases, respectively. Additivity was found between maximal effective concentrations of ET-1 and PMA. However, in vivo studies carried out in the rat, consistently showed a marked mitogenic effect of ETs exclusively addressed to the ZG. These findings agree with the "cell migration theory" of adrenocortical cytogenesis, according to which ZG is the locus of cell renewal under both basal and stimulated conditions (for review, see Nussdorfer, 1986). Mazzocchi et al. (1992) demonstrated that the s.c. infusion for 24 h of ET-1 (30 pmol  $\cdot$  min) evoked a 9-fold increase in the mitotic index (percentage of metaphase-arrested cells) of rat ZG. The infusion with equimolar doses of ACTH, ANG-II, and AVP raised the mitotic index 13-, 9-, and 10-fold, respectively. Combined infusion with ET-1 and ACTH increased the mitotic index by about 20-fold, whereas no additivity was observed between the effects of ET-1 and ANG-II or AVP. The ZG mitogenic effect of ET-1 was confirmed in both adult (Belloni et al., 1996b) and 19- or 38-day-old rats (Markowska et al., 1998). Mazzocchi et al. (1997), by using the in situ adrenal perfusion model, clearly demonstrated that ET-1 in-

creased the mitotic index and DNA synthesis in rat ZG cells. The maximal effective concentration was  $10^{-8}$  M (6- and 3-fold rises, respectively). ET-2 and ET-3 ( $10^{-8}$  M) exerted a similar effect as ET-1; however, the rank potency was ET-1 > ET-2  $\gg$  ET-3. The discrepancies existing between the findings of Mazzocchi et al. (1997) and Cozza and Gomez-Sanchez (1990) can be explained not only by assuming obvious interspecies variability of the response to ET-1 (calf versus rat), but also by taking into account that significant differences occur in the physiology of adrenocortical cells between in vivo conditions and in vitro culture. For instance, ACTH, which exerts a potent mitogenic action on the adrenal cortex in vivo, induces the functional differentiation of adrenocortical cells cultured in vitro, but simultaneously inhibits their proliferative activity (Nussdorfer, 1986).

b. REGENERATING ADRENALS. Two experimental models of adrenal regeneration were employed: in situ regeneration after enucleation and contralateral adrenalectomy (Nickerson et al., 1969) and autotransplantation of adrenal tissue fragments into bilaterally adrenalectomized rats (Belloni et al., 1990).

**i. Cell Hypertrophy and Steroidogenic Capacity.** Two s.c. injections of 2 nmol/kg ET-1, 12 and 24 h before the sacrifice, raised both PAC and PBC in rats bearing regenerating adrenals 4, 6, and 8 days after surgery (Malendowicz et al., 1997a,b). Vendeira et al. (1996a,b) observed that ET-1 raised PAC and PBC in autotransplanted rats within 60 min and markedly increased the volume of the mitochondrial compartment and SER in the ZG-like cells of regenerating tissue, a finding suggesting enhanced steroidogenic capacity.

**ii. Cell Proliferation.** ET-1 was found to evoke a marked increase in the mitotic index of enucleated regenerating adrenals (Malendowicz et al., 1997a; Ziele-niewski, 1997). Indirect evidence suggested a mitogenic effect of ET-1 also in adrenal autotransplants (Vendeira et al., 1996a,b). Adrenal fragments were implanted under the skin and observed 90 days after surgery. The large part of regenerated cells were of the ZF/R phenotype because they were provided with the IZAg antigen (Ho et al., 1994). Only a few clusters of subcapsular ZG-like cells were deprived of this antigen, as demonstrated by their negativity to the IZAb monoclonal antibody. ET-1 (0.5  $\mu\text{g}/\text{kg}$ ) markedly extended the subcapsular IZAb-negative zone, thereby indicating ZG-like cell proliferation.

2. *Testis.* To the best of our knowledge, no investigations have been carried out on the possible growth effect of ETs on normal Leydig cells. In the ET-1-responsive murine MA-10 Leydig tumor cell line, ET-1 concentration-dependently (from  $10^{-9}$  to  $10^{-6}$  M) increased gene expression of the *c-jun/c-myc* proto-oncogenes, but not the number of cultured cells (Ergul et al., 1993). This finding suggests a dissociation between the promoting effects of ET-1 on steroidogenic capacity and cell proliferation, again stressing that caution must be used in

interpreting the data obtained using in vitro cultured cells.

3. *Ovary*. a. FOLLICLE DEVELOPMENT AND CORPUS LUTEUM FORMATION. ET-1 concentration-dependently (from  $10^{-9}$  to  $10^{-7}$  M) stimulated proliferation of cultured human (Kamada et al., 1995) and pig granulosa cells (Kamada et al., 1993a; Kubota et al., 1994), the effect becoming evident after a 24- to 48-h exposure. In isolated ovaries from hypophysectomized estrogen-treated immature rats, within 4 h, ET-1 concentration-dependently stimulated DNA polymerase- $\alpha$  activity, but the nuclear DNA content per ovary was unchanged (Usuki et al., 1991c, 1992). These investigators advanced the hypothesis that in the rat ovary, ET-1 activates certain processes leading to DNA synthesis, the stimulation, however, not being high enough for complete activation of DNA duplication and mitosis. Furger et al. (1995) examined the effect of ETs on the FSH-induced "rounding-up" of human granulosa lutein cells cultured in vitro. Rounding-up is a phenomenon associated with the disaggregation of the cytoskeletal elements, and is the most rapid effect of growth agonists on cultured cells (e.g., ACTH and adrenocortical cells; for review, see Nussdorfer, 1986). Granulosa-lutein cells were cultured for 48 h and subsequently incubated for 1 h with FSH, which concentration-dependently raised the number of rounded-up cells. ET-1 (from  $10^{-12}$  to  $10^{-5}$  M) inhibited FSH-induced cell rounding-up; ET-3 was effective only at a micromolar concentration.

b. LUTEOLYSIS. In the calf, PGF $2\alpha$  is a luteotropic agent in young corpus luteum and a physiological luteolysin in the aged one; recent data indicate that ET-1 may participate in the PGF $2\alpha$ -induced luteolysis in vivo (Girsch et al., 1996a,b; Levin, 1996b; Meidan and Girsch, 1997; Ohtani et al., 1998). As surveyed above (Section VA), PGF $2\alpha$  was found to stimulate the release of ET-1 in the corpus luteum, where the peptide may reach local concentrations high enough to suppress progesterone secretion and to mediate, at least in part, the vascular events (dramatic decrease in blood flow and subsequent hypoxia) leading to corpus luteum regression.

#### B. Mechanisms of Action and Receptor Subtypes Involved

One mechanism that has been proposed to link hormone-receptor interaction with long-term trophic effect is the induction of transcription of regulatory proteins, which in turn modulate the activity of other regulatory genes, including those of the *fos/jun* family. Proto-oncogene proteins eventually mediate the growth effect of the hormones. Mitogen-activated protein kinase (MAPK) cascade is emerging as a pivotal pathway of these sequential responses. MAPKs are activated by most growth factors and can phosphorylate several different substrates, including proto-oncogene proteins, surely playing a major role in G $_0$ -G $_1$  and G $_2$ -M transition of the cell cycle. TK and PKC are essential in the activation of the above-described trans-

duction mechanisms (for review, see Wang et al., 1994; Malarkey et al., 1995). ETs have been found to activate not only TK and PKC, but also MAPK cascade, and to evoke a rapid increase in the mRNA levels of the members of the *fos/jun* gene family in many cell systems, including vascular smooth muscle cells (for review, see Rubanyi and Polokoff, 1994). However, evidence that ETs activate MAPK cascade in steroid-secreting tissues is not yet available.

1. *Adrenal Cortex*. a. ADULT AND JUVENILE ADRENALS. i. **Receptor subtypes.** The bulk of evidence strongly suggests that the ETA-receptor subtype mediates the growth and proliferogenic effect of ETs on rat ZG. Belloni et al. (1996b) and Markowska et al. (1998) showed that BQ-123, but not BQ-788, abolished the in vivo ZG proliferogenic effect of ET-1 in both adult and juvenile rats. Mazzocchi et al. (1997) confirmed this finding by using an in situ rat adrenal perfusion model. By dissolving the chemicals in the perfusion medium and perfusing adrenals for 180 min, they not only found that BQ-123 blocked ET-1 ( $10^{-8}$  M)-induced increase in ZG DNA synthesis, but also demonstrated that the proliferogenic action of ETs was directly related to their binding potency for ETA receptors (ET-1 > ET-2 > ET-3). Accordingly, specific agonists of ETB receptors (BQ-3020, IRL-1620, and STXb) were ineffective. The exclusive involvement of the ETA-receptor subtype in the ZG cell proliferogenic action of ETs raises some difficulties: in fact, although in the rat only ZG contains ETA receptors, ZG cells do not express ETA gene (see Section IVA). It may be tentatively conceived that only a small population of ZG cells, possibly the "stem cells" located just beneath the capsule (Nussdorfer, 1986), is provided with ETA receptors and that such population is lost during the mechanical procedures used to separate ZG from inner zones. Alternatively, it might be that ET-1, via ETA receptors located on nonparenchymal cells (fibroblasts, pericytes and/or endothelial cells), elicits the release of growth factors, which in turn stimulate the proliferation of ZG cells. However, endogenous ETs do not conceivably play a major role in the maintenance of adrenal growth, at least under normal conditions, because the prolonged treatment with BQ-123 alone did not alter the ZG mitotic index in adult rats (Belloni et al., 1996b; Mazzocchi et al., 1997). In addition, 4-week treatment of *Ren2* transgenic rats, a model of renin-dependent severe hypertension (for review, see Lee et al., 1996), with the ETA/ETB-mixed-receptor antagonist bosentan did not alter adrenal weight significantly (G.P.R., G.G.N., and G.M., unpublished results). Markowska et al. (1997) confirmed that basal ETA activation by endogenous ETs was not required for the maintenance of a normal ZG proliferative activity during the age-dependent adrenal growth, but reported the unexpected finding that BQ-788 administered alone doubled the ZG mitotic index, and that this effect was abolished by the simultaneous administration of BQ-123. These last observations may be tentatively explained by assuming that the occupancy of ETB recep-



tors by BQ-788 allows endogenous ETs to evoke an exceedingly high activation of ETA receptors, which in turn may be able to increase ZG cell proliferation.

**ii. Transduction mechanisms.** PKC and TK activation are common targets for ANG-II and ETs (see *Sections IIB* and *VB*), and the PKC- and TK-dependent activation of MAPK cascade has been reported recently to play a pivotal role in the adrenocortical proliferogenic effect of ANG-II (Schmitz and Berk, 1997; Tian et al., 1998). Mazzocchi et al. (1997) provided the direct demonstration that the ETA receptors, mediating the ZG proliferogenic effect of ETs in situ perfused rat adrenals, were coupled in the rat with the TK- and PKC-dependent signaling pathways. In fact, both the PKC-inhibitor Ro-31-8220 and the TK-inhibitor tyrphostin-23 reversed the proliferogenic effect of  $10^{-8}$  M ET-1, and when added together to the perfusion medium, abolished it. In contrast, the PKA inhibitor H-89, the CO inhibitor indomethacin, and the LO inhibitor phenidone were ineffective.

b. REGENERATING ADRENALS. BQ-788, but not BQ-123, prevented the rise in PAC and PBC induced by ET-1 in rats with enucleated/regenerating adrenals, thereby suggesting that the ETB receptor plays a major role in the mediation of the stimulatory action of ETs on adrenal steroidogenic capacity in this experimental model (Malendowicz et al., 1997b). The ET-1-induced rise in the adrenal mitotic index was blocked by nifedipine, which makes likely the possibility that it is dependent on the  $Ca^{2+}$  channel-mediated  $Ca^{2+}$  influx (Zielewski, 1997).

2. *Ovary.* The following findings strongly suggest the involvement of ETA receptors in the mediation of the growth effect of ETs in the ovary. ET-3 and IRL-1620 were ineffective in enhancing the proliferation rate of cultured bovine granulosa cells, and the proliferogenic effect of ET-1 was prevented by BQ-123 (Kamada et al., 1995). Likewise, BQ-123 abolished the inhibitory effect of ET-1 and ET-3 on FSH-induced rounding-up of human granulosa lutein cells (Furger et al., 1995). Moreover, the ETA receptor antagonist BQ-610 prevented the ET-1-mediated suppressive effect of PGF $2\alpha$  in bovine corpus luteum (Girsch et al., 1996a). ETA-receptor activation could conceivably be linked to the inhibition of adenylate cyclase cascade, inasmuch as this signaling mechanism is involved in the growth action of FSH on granulosa cells.

### C. Summary

Prolonged ET administration causes hypertrophy and increased steroidogenic capacity of adrenocortical cells and stimulates their proliferative activity. This last effect is specifically addressed to ZG cells and parenchymal cells of regenerating adrenocortical tissue. It seems to be exclusively mediated by ETA receptors, the signaling mechanism of which appears to involve the activation of PKC and TK, with the ensuing stimulation of MAPK-dependent cascade. No findings are available on

testis. In the ovary, ETs, mainly acting via ETA, are reported to favor ovarian follicle maturation and corpus luteum development. ETs, acting via ETA receptors, also are thought to play a crucial role in luteolysis.

## VII. Regulation of Endothelin Synthesis and Release in Steroid-Secreting Tissues

### A. Adrenal Gland

The large part of investigations was performed in this tissue, and the mechanisms whereby the local release of ETs could be regulated may include modifications of the blood flow and NO production. Although elevated shear stress has been reported to inhibit ET synthesis in vascular endothelium, evidence indicates that a moderate shear stress, like that generated by an increase in the blood flow rate, may elicit the release of ETs (*Section IIA*). ETs raise the release by vascular endothelium of NO, which, in addition to exerting a vasodilatory action, appears to have a role in terminating the response to ETs by altering ET receptor-mediated events (see *Sections IIA* and *VB*). The blockade of NOS results in a marked rise in ET-1 production by endothelium (Boulangier and Lüscher, 1990; Kuchan and Frangos, 1993), thereby indicating that NO decreases ET release and explaining why elevated shear stress, although markedly enhancing NO release, inhibits ET production in vessels.

1. *Increase in Blood Flow.* By using in situ perfused rat adrenals, a close direct correlation was observed between the flow rate of the perfusion medium and the concentration of ET-1 in the venous effluent. This direct correlation was present between 6 and 20 ml/10-min perfusion rate (Cameron and Hinson, 1993; Cameron et al., 1994). Lower perfusion rates (2 ml/10 min) did not change ET-1 release (Mazzocchi et al., 1998b). Obviously, any molecule capable of raising adrenal blood flow may also elicit intraglandular ET release. This, then, may explain their stimulatory effect on steroid secretion (see *Section VB*). Among these molecules there is ACTH, whose adrenal vasodilator effect is well known (Vinson et al., 1992). The bolus injection into the perfusion medium of 300 fmol of ACTH increased medium flow rate by 60% and ET-1 release by rat adrenal glands by 80% (Hinson et al., 1991b,c; Cameron et al., 1994). The injection into the perfusate of the mastcell degranulator compound 48/80 mimicked the effect of ACTH, whereas the injection of the mastcell granule stabilizer disodium cromoglycate blocked the ACTH effect (Hinson et al., 1991c). These investigators hypothesized that ACTH-induced adrenal hyperemia may be mediated by the release of histamine by local mast cells, and the ensuing increase in the intra-adrenal concentration of ETs may at least partly mediate the effects of ACTH on the adrenal cortex. Be that as it may, the finding that ACTH raised intra-adrenal release of ET-1 may explain the following rather intriguing observation of Markowska et

al. (1997): both BQ-123 and BQ-788 partially reversed the mitogenic effect of a 3-day administration of ACTH on rat zona glomerulosa.

2. *Effect of Nitric Oxide.* The addition of  $10^{-3}$  M L-arginine to the perfusion medium, although enhancing medium flow rate, inhibited by about 40% ET-1 release by in situ perfused rat adrenals (Hinson et al., 1996b). Conversely, the addition of  $5 \times 10^{-3}$  M L-NAME increased by about 2.5-fold ET-1 concentration in the venous effluent. This inhibitory effect of NO is unlikely to be attributable to an increase in cGMP production, inasmuch as the addition to the perfusion medium of 100  $\mu$ M sodium nitroprusside increased both flow rate (75%) and ET-1 release (90%); moreover, L-arginine lowered by 50% cGMP concentration in the perfusate. The mechanism(s) by which NO impairs ET release in adrenals remain(s) to be elucidated. However, the above surveyed findings raise the interesting possibility of the existence in the rat adrenals of negative feedback mechanisms involved in the fine tuning of ET release, which could have relevance in the regulation of adrenal blood flow and steroid secretion (Fig. 5).

#### B. Testis

It has been reported that cultures of Sertoli cells, but not Leydig cells, obtained from juvenile (20-day-old) rats released ET-1 under FSH control (Fantoni et al., 1993). Leydig cells from adult rats became positive to ET-1, and the s.c. injection of 50 IU hCG raised the intratesticular concentration of ET-1-LI by about 2-fold within 48 h (Collin et al., 1996). However, these last findings did not allow us to ascertain the source of hCG-inducible ET-1.

#### C. Ovary

As surveyed above (see Section III, A and B), ETs are synthesized and released in the mammalian ovaries. Magini et al. (1996) observed that the ET-1 concentration in the follicular fluid obtained from women undergoing gonadotropin stimulation was about 4-fold higher than in nontreated group. Usuki et al. (1991b) reported a very elevated concentration of ET-1 in the corpora lutea of experimentally superovulated rats. These results collectively suggest that gonadotropins may play a pivotal role in the modulation of ET production in the ovary. PGF $2\alpha$  also elicited ET release by corpus luteum (see Section VB), and it is conceivable that the rise in the local blood flow may be involved in this effect.

#### D. Summary

The increase in the shear stress on the vascular wall, caused by the physiological rise in the blood flow, enhances ET release in the adrenals. ET-induced NO production inhibits ET release, but increases blood flow. Blood flow and ETs enhance adrenal steroid hormone release, and NO probably decreases it: hence, the existence of local mechanisms involved in the fine tuning of adrenocortical secretion may be postulated. Gonadotro-

pins raise ET release in both the testis and ovary, thereby confirming the involvement of these peptides in the regulation of the ovary cycle. PGF $2\alpha$  stimulates both luteolysis and the release by corpus luteum of ETs, which in turn favor corpus luteum regression: the existence of close interrelationships between ETs and PGs in the luteolysis has been suggested.

### VIII. Involvement of Endothelins in the Pathophysiology of Steroid-Secreting Tissues

#### A. Adrenal Gland

1. *Aldosterone-Producing Adenomas.* a. **ENDOTHELIN SYNTHESIS.** Evidence of local synthesis of ET-1 in APAs was first provided by Imai et al. (1992), who found the expression of ppET-1 mRNA by Northern blotting analysis in homogenates of two tumors obtained from patients with Conn's syndrome. This finding was confirmed by Rossi et al. (1994, 1995b) with RT-PCR, which, being more sensitive, allowed the study of a large number of APAs. The possibility of the local synthesis of ET-1 was also supported by the results of immunocytochemistry. Incubation of 5- $\mu$ m-thick deparaffinized sections of eight APAs, 87% of which were small (<2 cm diameter), with a polyclonal rabbit anti-human ET-1 antiserum allowed Li et al. (1994, 1999) to show that about 50% of the tumor cells exhibited a moderate staining; the immunoreactive products were similar to those found in the adjacent tumor-free cortex (see Section IIIB), but they were generally fewer in number per cell. The lower expression of ET-1/big ET-1 gene in APAs than in normal adrenals recently was confirmed immunocytochemically (Hiraki et al., 1997). The observation that APAs expressed ECE-1 gene (Rossi et al., 1995a) fully agrees with the concept of local ET-1 biosynthesis in these tumors. Moreover, it has been found that plasma levels of ET-1 were significantly higher in patients with APA than in normal subjects (Letizia et al., 1996), which may be interpreted as an epiphenomenon of the elevated ET-1 synthesis in the tumors.

b. **ENDOTHELIN RECEPTORS.** Imai et al. (1992) demonstrated the presence of ETA and ETB receptor subtype mRNAs in two APAs; however, experiments with  $^{125}$ I-ET-1 saturation and competitive displacement binding by different ET isopeptides were compatible with the existence of only a single class of receptors. Conclusive evidence of the occurrence of both ET receptor subtypes in APAs was provided by Rossi et al. (1995b) with gene expression studies and  $^{125}$ I-ET-1 competitive displacement binding by ET-3, BQ-123, and STXc, and with autoradiography. However, marked differences in ETA receptor density between ZG cell-like and ZF cell-like tumors and even among histologically different areas of the same tumor were noted. Hiraki et al. (1997) observed by immunocytochemistry much more abundant ETB than ETA receptors in APAs.

c. EFFECTS OF ENDOTHELINS ON HORMONAL SECRETION. In an *ex vivo* study of adrenocortical tissue slices, ET-1 was found to enhance aldosterone secretion, although less potently than ANG-II, from normal adjacent adrenal tissue, but not from APA tissue (Zeng et al., 1992). These investigators suggested the possibility of down-regulation of responsiveness to ET-1, attributable to the increased local ET-1 synthesis in the tumors. However, opposite results were obtained by using dispersed cells from nine ANG-II-responsive APAs (G.G.N. and G.P.R., unpublished results). ET-1 ( $10^{-9}$  M) significantly raised pregnenolone, progesterone, corticosterone, 18OH-corticosterone, and aldosterone production, without affecting 11-deoxycortisol, cortisol, and 11-deoxycorticosterone yield, thereby suggesting that ET-1 activated both early and late steps of aldosterone synthesis (Fig. 3). Like in normal human adrenals (see *Section VB*), both ETA and ETB receptors mediated the aldosterone secretagogue effect of ET-1 because aldosterone response was partially reversed by either BQ-123 or BQ-788 ( $10^{-7}$  M) and abrogated by the simultaneous exposure to both antagonists.

2. *Glucocorticoid- and Sex Steroid-Secreting Tumors.* The role of ETs has not been extensively investigated in patients with Cushing's syndrome or virilization secondary to adrenal adenomas, mainly attributable to the fact that, although glucocorticoids can influence ET-1 synthesis in vascular smooth-muscle and pulmonary epithelial cells (Kanse et al., 1991; Calderon et al., 1994; Provencher et al., 1995), the opposite does not seem to be true (Kennedy et al., 1993). Li et al. (1994) reported that about 50% of the cells of six medium-sized (from 2 to 5 cm in diameter) adrenocortical adenomas obtained from patients with Cushing's syndrome displayed an immunocytochemically demonstrable, moderate ET-1 staining. The immunoreactive products were similar to those found in APA and nonfunctioning adenoma cells. Hiraki et al. (1997) observed that tumor cells of 32 asymptomatic or Cushing's syndrome-producing adenomas exhibited ET-1/big-ET-1-LI and ETB receptor-LI and less-abundant big-ET-3/ETA receptor-LI. Differences between hyperfunctioning and nonfunctioning adenomas were not appreciable.

3. *Adrenocortical Carcinomas.* No immunoreactive ET-1 staining was found in two large (about 5-cm diameter) virilizing carcinomas, but in a number of nonfunctioning carcinomas, ET-1-stained cells ranged from none to less than 10% (Li et al., 1994, 1999). These findings allowed these investigators to advance the hypothesis that ET-1 immunostaining may be of diagnostic value for identification of malignancy. However, according to Hiraki et al. (1997), carcinomas displayed ET-1/big-ET-1-LI and ETB receptor-LI similar to adenomas. Rossi et al. (1997a) and Mathieu et al. (1998) demonstrated by RT-PCR both the ppET-1 and ECE-1 mRNAs in a cell line of adrenocortical cancer (NCI-H295), which secretes steroid hormones in a regulated fashion (Gazdar et al., 1990; Bird et al., 1993, 1994, 1996; Rainey et al., 1994).

In these cells, the presence of ET-1 was also detected by immunocytochemistry (Rossi et al., 1997a), and NCI-H295 cell cultures were found to release ET-LI in the medium in a time-dependent fashion (Mathieu et al., 1998). NCI-H295 cells also expressed both ETA and ETB receptor subtypes at the mRNA and protein level (Rossi et al., 1997a). These last investigators also demonstrated by semiquantitative RT-PCR that both ET-1 and IRL-1620 raised aldosterone synthase gene expression in this cell lines, an effect which was decreased, although not abolished, by either BQ-123 or BQ-788. ET-1 ( $10^{-8}$  M) and IRL-1620 ( $10^{-7}$  M) acutely increased  $[Ca^{2+}]_i$  in NCI-H295 cells, and the effect was blocked by a 10-min pretreatment with BQ-788, but not BQ-123. Collectively, these findings suggested that: 1) NCI-H295 cells possess an active ET-1 biosynthetic pathway; and 2) ET-1 regulates in an autocrine/paracrine fashion aldosterone secretion of these cells by enhancing both aldosterone synthase transcription and  $[Ca^{2+}]_i$ , the former effect being mediated by both receptor subtypes, and the latter one only by the ETB receptor.

4. *Idiopathic Hyperaldosteronism.* The involvement of ETs and their receptor subtypes in idiopathic hyperaldosteronism has not been investigated thus far, mainly because of the lack of adrenocortical tissue suitable for molecular and pharmacological *in vitro* studies. The plasma levels of ET-1 were not found to be increased compared to normal subjects in seven patients with primary aldosteronism with no evidence of adrenal tumor using computer tomography scanning (Veglio et al., 1994).

#### B. Testis

To our knowledge, no investigations are at present available on the possible role of ETs in the pathophysiology of Leydig cells. Only one study showed that ETs may control the growth of murine Leydig cell tumor MA-10 cell line (see *Section VIA*).

#### C. Ovary

No specific investigations were carried out which were aimed at ascertaining any possible involvement of ETs in the pathophysiology of the mammalian ovary. However, it has been suggested that ETs could play a role in modulating ovarian circulation in pathological conditions such as ovarian hyperstimulation syndrome, which is known to be associated with elevated estrogen concentration and a high number of active follicles (Haq et al., 1996). Moreover, ET-1 was found to induce a rapid *c-fos* expression and to promote proliferation of ovarian carcinoma cells cultured *in vitro*, acting through PLC- and TK-coupled ETA receptors (Bagnato et al., 1997). This finding lends support to the view that ETs may act as autocrine factors involved in the pathogenesis of ovarian carcinomas (Bagnato et al., 1995).

## IX. Conclusions and Perspectives

The preceding sections of this survey have shown that in the 10 years since the discovery of ETs, an impressive mass of data has been accumulated which clearly shows that these peptides play potentially important roles not only in the functional regulation of the cardiovascular system (Lüscher and Lerman, 1998; Miyauchi and Masaki, 1999), but also in the autocrine/paracrine modulation of secretion and growth of steroid-secreting tissues. ETs are synthesized and released in the steroid-secreting tissues, the parenchymal cells of which are provided with specific ET receptors. ETs were shown to: 1) enhance mineralocorticoid and glucocorticoid release by mammalian normal and tumorous adrenocortical cells, acting through both ETA and ETB receptors, and to exert a marked proliferogenic effect on ZG cells, exclusively via the activation of the ETA receptor subtype; 2) stimulate testosterone production by testis Leydig cells via ETA receptors; and 3) inhibit agonist-stimulated estrogen and progesterone secretion of mammalian ovary, to stimulate the proliferation of granulosa cells of ovarian follicles, and to favor corpus luteum formation and regression, all of these effects being mediated by ETA receptors.

These effects of ETs were largely demonstrated by in vitro studies and need to be confirmed by further investigations carried out in vivo. In fact, only the demonstration that short- or long-term administration of ET-receptor antagonists and/or ECE inhibitors is able to elicit sizeable effects on steroid-secreting tissues could provide conclusive evidence of the involvement of endogenous ET systems in the functional control of these tissues under either physiological or pathophysiological conditions. Unfortunately, this kind of investigation gave elusive results so far, probably because of the intrinsic difficulties of in vivo experimentation. In fact, the wide distribution and multiple activities of ETs in several organs and systems, the primary functions of which are strictly connected with those of steroid-secreting tissues (e.g., hypothalamo-pituitary axis, kidney and extra-kidney renin-angiotensin system, and atrial myocardium; for references, see Bumpus et al., 1988; Gutkowska et al., 1993; Saez, 1994; Li et al., 1995b; Stojilkovic and Catt, 1996), as well as the multiple interrelationships existing between adrenals and gonads (e.g., glucocorticoids depress testosterone secretion and sex hormones affect glucocorticoid production; for references, see Nowak et al., 1995; Michael et al., 1996; Hardy and Ganjam, 1997), could make it difficult to unequivocally interpret the results of in vivo studies.

Apart from the above-mentioned need of in vivo studies with appropriate experimental models, we shall now take the opportunity to mention some topics whose elucidation could notably increase our understanding in this field. First, are the expression of ET, ECE, and ET receptors altered under physiological or pathophysiological

conditions requiring or causing enhanced or decreased steroid hormone secretion? This occurs for other intra-adrenal regulatory peptides. For instance, the content of ANP protein and mRNA is higher in adrenals of human patients with primary aldosteronism than in normal glands obtained from kidney donors (Lee et al., 1993, 1994). Second, might some dysfunctions of the ovulatory cycle be caused by alterations of the local ET system? Third, does alteration of the adrenal ET system play a role in the pathogenesis of some form of aldosterone-dependent hypertensive diseases? In this connection, the possible interactions of ETs with the adrenal adrenomedullin system merit exploration. In fact, adrenomedullins are potent aldosterone-antiseoretagogue hypotensive peptides synthesized in adrenal medulla (for review, see Nussdorfer et al., 1997b), and ETs have been found recently to elicit adrenomedullin secretion by vascular endothelium (Jougasaki et al., 1998), and, conversely, adrenomedullin inhibits ET-1 production by vascular smooth muscle cells (Kohno et al., 1995). Fourth, because of their potent proliferogenic actions, are ETs involved in the growth (and perhaps in the pathogenesis) of some kinds of tumors of the steroidogenic tissues (e.g., APAs and ovarian carcinomas). A role for ETs as autocrine regulators of tumor cell growth has been recently suggested (Bagnato and Catt, 1998). Finally, are there interrelationships between the gonadal ET system and the pathophysiology of some sex steroid-dependent tissues like mammary gland and prostate? In this connection, it is of interest to recall that evidence suggests the involvement of ETs in the development of breast and prostate tumors (Nelson et al., 1996; Patel et al., 1997) and prostatic hyperplasia (Wu-Wong et al., 1997; Walden et al., 1998).

The answer to these and many other basic questions, including the not-yet-settled problem of multiple mechanisms of action and signaling mechanisms of ETs, along with the continuous development of new, potent, and selective antagonists of ET receptors and ECE inhibitors, will not only increase our knowledge of steroid-secreting tissue physiology, but also will open novel and important perspectives in the therapy of adrenal and gonadal diseases.

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