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Pharmacology of Cotransmission in the Autonomic Nervous System: Integrative Aspects on Amines, Neuropeptides, Adenosine Triphosphate, Amino Acids and Nitric Oxide[a]

JAN M. LUNDBERG

Research and Development, Astra AB and Department of Physiology and Pharmacology, Karolinska Institute, Stockholm, Sweden

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Address for reprints and correspondence: Research and Development, Astra AB, S-151 85 SÖDERTÄLJE, Sweden.

Abbreviations: CNS, central nervous system; NA noradrenaline; ACh, acetylcholine; DMPP, 1,1-dimethyl 4-phenylpiperazinium; NANC, nonadrenergic, noncholinergic; EDRF, endothelium-derived relaxant factor; IP_3, inositoltrisphosphate; cAMP, cyclic adenosine monophosphate; NPY, neuropeptide Y; VIP, vasoactive intestinal polypeptide; LDV, large, dense-cored secretory vesicles; SP, substance P; mRNA, messenger ribonucleic acid; ATP, adenosine 5′-triphosphate; EPSP, excitatory postsynaptic potentials; EJP, excitatory junction potentials; NO, nitric oxide; NOS, nitric oxide synthase; EDRF, endothelium-derived relaxing factor; cGMP, cyclic guanosine monophosphate; Hb, hemoglobin; L-NMMA, N^G-mono-methyl-L-arginine; CO, carbon monoxide; PG, prostaglandin; HO, heme oxygenase; Zn PP, zinc-protoporphyrin-IX; RTX, ^{3}H-resiniferatoxin; NGF, nerve growth factor; TTX, tetrodotoxin; CTX, ω-conotoxin; COX, cyclo-oxygenase; PG, prostaglandin; PGI_2, prostacyclin; TK, tachykinin; PDE, phosphodiesterase; NKA, neurokinin A; NKB, neurokinin B; NPK, neuropeptide K; NPγ, neuropeptide γ; PPT I, preprotachykinin I; NEP, neutral endopeptidase-24.11 (EC.3.4.24.11); ACE, angiotensin-converting enzyme (EC 3.4.15.1); CGRP, calcitonin gene-related peptide; EFS, electrical field stimulation; NADPH, reduced nicotinamide-adenine dinucleotide; KA, kainate; NMDA, N-methyl-D-aspartate; AMPA, alpha-amino-methyl-isoxazole-proprionic acid; PHI, peptide with N-terminal histidine and C-terminal isoleucine (in rat and pig); PHM, the counterpart of PHI in man; PACAP, pituitary adenylate cyclase activating peptide; GTP, guanosine triphosphate; L-NNA, N^G-nitro-L-arginine; L-NAME, L-nitro-arginine-methyl-ester; IJP, inhibitory junction potential; 5′-triphosphatase, ecto-ATPase.

I. Introduction

A. Classical Autonomic Neurotransmission

The autonomic nervous system plays an important role in health and disease. In the healthy organism, autonomic control of a variety of organ systems serves to maintain homeostasis. Visceral and somatic afferents convey essential information to autonomic centers in the central nervous system (CNS), which in turn trigger appropriate reflex adjustments via autonomic preganglionic and postganglionic motor nerves. Environmental stimuli can also excite central autonomic centers. Drugs that modulate autonomic transmission have been in use for years, and among them are some of the oldest drugs known to humans.

The peripheral autonomic nervous system is subdivided into the sympathetic and parasympathetic parts, which use noradrenaline (NA) (von Euler, 1946, 1948) and acetylcholine (ACh) (Loewi and Navratil, 1926) as their main respective postganglionic chemical transmit-

ters. In preganglionic nerves, ACh serves as transmitter in both sympathetic and parasympathetic ganglia. In fact, two classes of cholinergic receptors were initially defined early in this century on the basis of tissue responses to certain naturally occurring agonists and antagonists (Dale, 1914). Muscarinic receptors were defined in a tissue as a response similar to the effect of muscarine (an alkaloid derived from the poisonous mushroom *Amanita muscaria*) and antagonized by atropine (an alkaloid from the deadly nightshade, *Atropa belladonna*). Similarly, ganglionic nicotinic receptors were defined as a response similar to the effect of nicotine (a well known constituent of tobacco) and blockade by D-tubocurarine (one of the alkaloids from the South American arrow poison). More than a century ago, even before the transmitter concept was established, it was noted that substances such as atropine, which block cholinergic muscarinic transmission, did not eliminate all the functional responses to parasympathetic nerve stimulation of salivary glands (Heidenhain, 1872). The existence of other vasodilatory factors released with ACh was postulated (Hilton and Lewis, 1956) but could not be proven until the late 1970s, when histochemical techniques made it possible to show colocalization of classical transmitters and vasodilatory peptides in autonomic parasympathetic nerves (Lundberg et al., 1979; Lundberg et al., 1980). Many drugs have been developed that interfere with autonomic transmission prejunctionally or postjunctionally, and these have been widely used during the last decades, both experimentally and to treat a variety of clinical conditions. It has become increasingly evident that these drugs do not influence classical transmitters exclusively: they also exert complex actions on other mediators involved in the cotransmission process.

The chemical transmission of autonomic nerve signals is thus likely to involve a variety of agents that often are released from the same nerve, a principle present also in lower species (Kupfermann, 1991). Distinct patterns of synthesis, storage, release and degradation of these agents and of receptor expression thus form a basis for complex cotransmission. The term "neurotransmitter" in the present review refers to "any substance secreted by a neuron to control its target cells," even though it is recognized that stringent evidence for transmitter function is absent in some cases because of lack of potent and selective antagonists (Potter et al., 1981).

The neuronal control of effector cells may involve both small and large molecules exerting excitatory or inhibitory effects lasting for a relatively short period, on the order of milliseconds to minutes. It should also be emphasized that for a variety of neuropeptides, more long-term trophic actions are also apparent. Finally, there are considerable species variations in autonomic neurotransmission mechanisms, and the examples given here often refer to species, tissues or organs in which typical responses are present. This review will also discuss those instances in which studies indicate possible clinical relevance in humans or suggest target principles for pharmacological development.

This integrative review is focused on pharmacology of nonadrenergic, noncholinergic (NANC) mechanisms in the motor and sensory components of the autonomic and enteric nervous system and on how these "new" transmitters or transmitter candidates interact with classical adrenergic, cholinergic and glutamatergic functions. Initially, a brief summary of recent aspects on classical transmitter receptor mechanisms is given.

1. Acetylcholine. In the past years, important advances have been made in understanding cholinergic receptor pharmacology. Nicotinic ACh receptors (N_1 = skeletal muscle type and N_2 = ganglionic type) are ligand-gated Na^+ channel receptors whereby ACh binds to a major extracellular domain of the protein (Deneris et al., 1991; Changeux et al., 1992). The N_2 receptors found, for example, in autonomic ganglia are preferentially activated by 1,1-dimethyl-4-phenylpiperazinium (DMPP) and inhibited by classical ganglion blockers such as hexamethonium or chlorisondamine.

In 1986, Numa and colleagues (Kubo et al. 1986a,b) cloned the m1 and m2 subtypes of the muscarinic receptor. Muscarinic agonists require a positive charge to be active (Burgen, 1965); using point mutations, it was demonstrated that an aspartic acid residue (aspartic acid 105) in the transmembrane segment III of the muscarinic receptor is highly conserved and provides a negative charge for ligand binding of ACh (Fraser et al., 1989). Five distinct molecular forms of the G-protein-coupled muscarinic receptors have now been cloned, and three of these subtypes have been pharmacologically characterized (Kubo et al., 1986a, b; Bonner et al., 1987; Goyal, 1989). Selective agonists and antagonists are now available for the m1, m2 and m3 subtypes. Thus, the m1 receptor is a classical ganglionic neural type of receptor that facilitates the ganglionic transmission after a nicotinic stimulus and is antagonized by pirenzepine. M_2 receptors are prejunctional on cholinergic and adrenergic nerves. These receptors provide negative cholinergic feedback on transmitter release. M_2 receptor agonists inhibit—and specific antagonists, such as gallamine, potentiate—nerve stimulation-evoked ACh release (Fryer and Maclagan, 1984). M_3 receptors (*a*) cause contraction of visceral smooth muscle in the respiratory, gastrointestinal and urogenital tract, (*b*) stimulate secretion from exocrine gland cells, such as salivary glands and (*c*) mediate production of endothelium-derived relaxant factor (EDRF) (Goyal, 1989; Duckles and Garcia-Villalon, 1990). It should be emphasized that the classical antagonist atropine has similar affinity for all three subtypes of muscarinic receptors (Goyal, 1989). Muscarinic m1 and m3 receptors are coupled to generation of inositol-trisphosphate (IP_3), whereas m2 receptors inhibit cyclic adenosine monophosphate (cAMP) formation and influ-

ence K^+ and Ca^{2+} channels, thereby inhibiting cardiac muscle contractility and autonomic transmitter release.

2. Noradrenaline. Adrenergic receptors were classified as α-adrenoceptors and β-adrenoceptors nearly half a century ago (Ahlquist, 1948). Some years later, Langer (1974) suggested a subclassification of α-adrenoceptors into α_1 and α_2 subtypes, located postjunctionally and prejunctionally, respectively, whereby α_2-receptors regulate NA release (Farnebo and Hamberger, 1971). α_2-Adrenoceptors have since been shown also to be present postjunctionally, e.g., on vascular smooth muscle (Docherty and Hyland, 1985; Docherty, 1989). Several subtypes of α_1-, α_2- and β-receptors have now been characterized (see Bylund et al., 1994). Adrenoceptors of the β-type were purified by Vauquelin et al. (1977), and the first complete sequence of the β_2-receptor was reported using molecular cloning techniques (Dixon et al., 1986). Adrenergic receptors possess several important properties; they are able to bind agonists and antagonists with a characteristic specificity. Mutation studies using chimeric receptors indicate that several membrane-spanning domains of α-adrenergic and β-adrenergic receptors are involved in this binding. Presumably, adrenergic agonist and antagonists intercalate in the central pocket (isosterically) and interact with amino acid residues belonging to some of the membrane-spanning domains of the receptor (Frielle et al., 1988; Kobilka et al., 1988; Lefkowitz et al., 1989; Lomasney et al., 1991). A specific and direct interaction of the amine group of the ligand with the carboxylic group of a conserved aspartic acid residue in transmembrane segment III has been substantiated in great detail through combined use of receptor protein engineering by molecular biological techniques and medicinal chemistry (Strader et al., 1991). The pharmacology of these receptors has not yet been completely clarified, and especially prazosin, initially purported to be a selective α_1-antagonist, also inhibits α_{2B}-adrenoceptors (Ruffolo et al., 1991). α_1-Adrenoceptors are generally coupled with IP_3 turnover, whereas α_2-adrenoceptors reduce cAMP production and influence K^+ and Ca^{2+} channels (Bylund, 1988). Additional evidence also supports the existence of a β_3 receptor (Emorine et al., 1989). All β-receptors seem to be coupled to adenylate cyclase, stimulating cAMP production. For recent details on adrenoceptor mechanisms, see Bylund et al. (1994).

B. Nonadrenergic, Noncholinergic Autonomic Motor and Sensory Transmission

Until the late 1970s, chemical transmission from autonomic nerves to their peripheral effector tissues generally had been thought to be mediated by NA or ACh. Increasing evidence was accumulating that some autonomic neurons utilize transmitter mechanisms unrelated to ACh or NA and represented separate entities, so-called "purinergic nerves" (Burnstock, 1972). This NANC transmission concept has been obvious, especially regarding the autonomic control of both visceral and vascular smooth muscle.

In spite of pharmacological blockade of adrenergic receptors, sympathetic nerve activation still evokes residual vasoconstriction in several vascular beds in experimental animals (Folkow and Uvnäs, 1948; Lundberg and Tatemoto, 1982) and in humans (Taddei et al., 1989). Furthermore, electrical nerve stimulation evokes contraction of isolated blood vessels in vitro, even in the presence of adrenoceptor blocking agents (Bevan and Su, 1971; von Kügelgen and Starke 1985; Burnstock and Kennedy 1986; Morris and Murphy, 1988; Morris, 1991). Similarly, the classical muscarinic receptor antagonist atropine blocks the effects of exogenous ACh but only marginally influences the parasympathetic nerve-mediated vasodilation in organs such as the submandibular salivary gland (Heidenhain 1872; Lundberg 1981).

Traditionally, the sensory nerves have been thought to function as a receptive system that reacts to changes in the environment or internal organs and reflexogenously activates autonomic motor nerves via the CNS and enables the organism to maintain homeostasis. However, more than a century ago, it was found that "antidromic" stimulation of the peripheral stump of transected dorsal roots of sensory nerves (i.e., in the opposite direction of the orthograde propagation of the nerve impulse to the CNS) induced vasodilation and other signs of inflammation in the skin (Gaertner, 1889) (fig. 1). Thus, the vasodilatory flare component of the classical triple response reaction (e.g., to allergen in the skin of humans) is a local axon reflex mechanism mediated by mast cell products, such as histamine, triggering release of NANC sensory transmitter(s) (Lundblad et al., 1987a). Therefore, it is clear that afferent neurons not only play a role in sensory transmission but also take part in local effector systems, such as in inflammatory responses to tissue irritation and injury (Bruce, 1910; Chapman and Goodell, 1964; Foreman and Jordan 1984; Jansco et al., 1967; Lembeck, 1983; Szolcsanyi, 1988). This is sometimes referred to as the "motor" function of nocifensor sensory nerves. Furthermore, such a local effector role of sensory nerve endings may also involve control of visceral smooth muscle, heart muscle, transmission in autonomic ganglia, immunological processes and, finally, tissue growth and repair (Lundberg and Saria, 1987; Holzer, 1988) (fig. 1).

A major development in the field of sensory neurons has been based on the use of capsaicin, the pungent ingredient in a number of red peppers of the genus *Capsicum*. This compound selectively activates certain afferent neurons (A-δ and C-fibers) followed by neurotoxicity. Stimulation of afferent neurons by capsaicin gives rise to irritant and painful sensations via central reflexes. In the airways, it activates protective reflexes such as sneezing, coughing and bronchoconstriction as well as avoidance and escape reactions (fig. 1, Gamse, 1982; Buck and Burks, 1986; Lundberg and Saria, 1987;

Holzer, 1991). Other reflexes upon capsaicin-induced stimulation of sensory neurons involve cardiovascular (Crayton et al., 1981; Lundblad et al., 1984), neuroendocrine (Mueller 1981; Watanabe et al., 1988) and thermoregulatory mechanisms (Rabe et al., 1980; Szallasi and Blumberg 1989). It should be emphasized that capsaicin can activate both sympathetic and parasympathetic autonomic reflexes. Thus, irritation of nasal C-fiber afferents causes local nasal mucosal vasodilation and exocrine secretion via parasympathetic mechanisms, but also systemic hypertension caused by sympathoadrenal activation (Lundblad et al., 1984; Stjärne et al., 1989a, b).

1. Neuropeptides. The discovery of potent neuropeptides such as neuropeptide Y (NPY) (Tatemoto, 1982) and vasoactive intestinal polypeptide (VIP) (Said and Mutt, 1970a, b; Mutt and Said, 1974) in postganglionic autonomic perivascular nerves (Lundberg et al., 1979, 1980a, 1982d) gave new insight into the possible mediator mechanisms in neurogenic vascular control, especially for long-lasting responses (Lundberg and Hökfelt, 1983). Even if NA and ACh represent general transmitters in sympathetic and parasympathetic nerves, peptides such as NPY and VIP occur in subpopulations of autonomic neurons, thus allowing further specialization of autonomic function. Thus, NPY is present in adrenergic cardiovascular nerves but not in NA fibers to exocrine gland cells or brown fat cells (see Lundberg et al., 1990). In analogy, VIP is present in sphenopalatine ganglion cells innervating exocrine glands and blood vessels but not in the ciliary ganglion providing cholinergic innervation of, for example, iris smooth muscle (Lundberg, 1981). In the enteric nervous system, there is an even further complicated chemical coding of neurons involving a variety of peptides and classical transmitters (Furness et al., 1989, 1992b) suggesting plurichemical transmission.

Neuropeptides are produced from large precursor molecules and often occur together in structurally related families. The major site of processing of neuropeptide precursors is in the secretory granules in which prohormone convertases cleave specifically at monobasic or dibasic amino acid residues within the precursor (Seidah et al., 1993). Additional processing through C-terminal amidation and carboxypeptidase action produces the final secreted form of the peptide. Neuropeptides are contained in large, dense-cored secretory vesicles (LDV) (fig. 1) that often discharge their contents at morphologically undifferentiated (i.e., nonsynaptic) sites (Thureson-Klein and Klein, 1990). Because of their slow rate of degradation, peptides can diffuse over a wide area to exert their often long-lasting actions. A wider radius of action from the release site also follows from the considerably higher affinities neuropeptides have for their receptors compared with, for example, amine transmitters. Some targeted release adjacent to postsynaptic cells is also likely to occur, however (see Bean et al., 1994; Golding, 1994). The stimulated exocytosis of LDV is different from that of small synaptic vesicles and may involve differential sensitivity to calcium, whereby neuropeptide release is triggered by elevations in the Ca^{2+} concentrations in the bulk cytoplasm, whereas secretion of amino acids stored in small vesicles require high local elevations as produced in the vicinity of Ca^{2+} channels—that is near active zones of synapses (Verhage et al., 1991). This could explain why, in many cases, substantial peptide release seems to occur mainly at high frequency stimulation, although this is not a general rule (Lundberg et al., 1994a). In some cases, the released peptide then might need to be further processed to generate full biological activity.

The peptide substance P (SP) has long been considered a major candidate for some of these NANC sensory responses (Hellauer and Umrath, 1948; Lembeck, 1953; Holton, 1959; Pernow, 1983) (fig. 1). The isolation and chemical sequencing of SP (Chang et al., 1971) and the subsequent demonstration that this peptide was present in sensory neurons (Hökfelt et al., 1975) supported its role as a NANC mediator. However, today it is clear that a variety of sensory neuropeptides must be considered in this respect. Thus, several members (tachykinins) and receptors (neurokinin (NK) receptors) for an SP family of peptides have been cloned (Nakanishi, 1991). Furthermore, the recently developed potent receptor antagonists that block the actions of exogenous SP only inhibit some sensory NANC responses. These small molecular peptide receptor antagonists of nonpeptide nature represent a major breakthrough in neurotransmission research and can in many cases, in contrast to peptides, pass the blood-brain barrier. These molecules have emerged from a combination of major screening effects and rational drug design. Also, antisense probes may be used as functional antagonists whereby oligo-nucleotide probes complementary to a sequence of certain peptide messenger ribonucleic acids (mRNAs), such as a neuropeptide receptor, have been demonstrated to attenuate translation of mRNA into protein; however, this technique has several drawbacks, including instability and lack of tissue penetration of the probes, and should be used mainly in the absence of a suitable antagonist (Wahlestedt, 1994).

The expression of neuropeptides may vary considerably during different conditions, including endogenous variations, after experimental procedures, during drug treatment or in pathophysiological states. This is to be expected, because peptides in general are assumed to be produced ribosomally and because replacement after release only seems to occur via new synthesis in cell bodies and axonal transport to terminal regions. The dramatic regulation of peptide synthesis has been particularly evident when using in situ hybridization techniques for analysis of peptide mRNA levels in neuronal somata under various pathophysiological states (see Hökfelt et al., 1994). In contrast, it is possible to keep classic transmitter levels relatively constant under various condi-

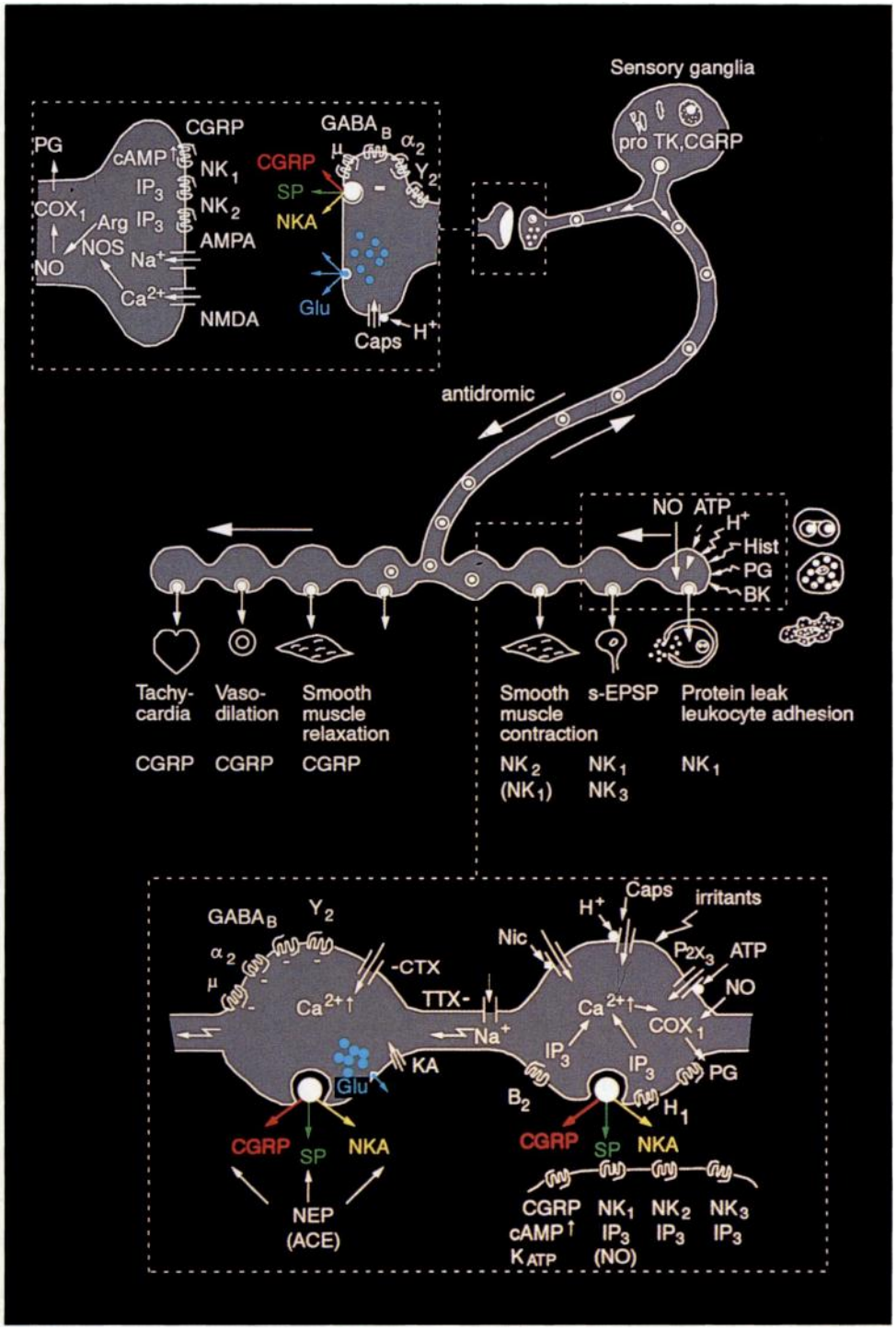

FIG. 1. Schematic illustration of a polymodal capsaicin-sensitive nocifensor sensory neuron containing several transmitters including various peptides (SP, NKA, and CGRP), and glutamate (Glu). From cell bodies in spinal and visceral (e.g., nodose) ganglia, peripheral branches project to a variety of organs and tissues with varicose arborizations close to different effector cells. This provides the anatomical basis for axon reflex responses via local peptide release upon irritants, ischemia, injury, inflammation, infection and mosquito bites. The central branch of the sensory neuron projects to the CNS, where it synapses with second order sensory neurons. In the central synapse (upper magnified part), glutamate is the main transmitter, potentially acting on a variety of receptors including AMPA and NMDA receptors. In addition, other mechanisms, especially NK_1 and NK_2 receptor mechanisms, may facilitate the central transmission process. Elevated Ca^{2+} may lead to both NOS and COX1 activation in the CNS with subsequent formation of NO and various PGs diffusing within the tissue. Activation of prejunctional μ-opioid, α_2- and $GABA_B$ receptors inhibits mediator release from central terminals of primary sensory neurons. Peripheral nociceptor neurons can be activated by a variety of exogenous irritants, such as cigarette smoke, acrolein, formalin, xylene and the hot pepper agent capsaicin, and by endogenous substances, such as bradykinin (BK), histamine (Hist), NO, protons (H^+) ATP, and various prostaglandins (PG), that are formed upon tissue injury or are released from inflammatory cells. This activation then leads to depolarization, action potential generation and propagation first to adjacent varicosities (axon reflexes) and finally to the CNS. It has traditionally been thought that these neurons mediate sensations such as irritation, itching and pain as well as protective reflexes: coughing

tions caused by local synthesis in nerve terminals and also replacement by efficient reuptake mechanisms close to release sites.

2. Adenosine 5′-triphosphate. Adenosine 5′-triphosphate (ATP), a well known constituent of transmitter storage vesicles (Fried, 1980), is a key candidate to mediate rapid and short-lasting excitatory postsynaptic potentials (EPSP), alternatively named excitatory junction potentials (EJP) in the absence of synaptic specialization, leading to contractile responses to electrical field stimulation of sympathetic nerves, especially those seen in blood vessels, vas deferens and urinary bladder (Fedan et al., 1981; Burnstock and Kennedy, 1986; Burnstock, 1986a, b, 1988, 1990). Initially, two classes of purinoceptors were described based on pharmacological criteria; at P_1 receptors, the ATP degradation product adenosine and its analogs were agonists, and their effects were blocked by various xanthines, such as theophylline. P_2 receptors were activated by ATP, and various more or less selective antagonists for these receptors have been postulated (Burnstock, 1978). The P_1/P_2 nomenclature has undergone continuous refinement as new receptor subclasses have been identified on the basis of pharmacological and molecular cloning studies (see Fredholm et al., 1994; Dahlziel and Westfall, 1994; Fredholm, 1995; Kennedy and Leff, 1995b).

3. Nitric oxide. The gas nitric oxide (NO) is not prestored as are other transmitters, but is synthesized upon demand from the terminal guanidino nitrogen atom of l-arginine by nitric oxide synthase (NOS) (Bredt and Snyder, 1992). NO release does not involve vesicles and exocytosis but relies on diffusion, which is a slower process. The enzyme responsible for neuronal NO synthesis is activated by Ca^{2+}, and the moment-to-moment control of the rate of synthesis depends on the intracellular Ca^{2+} concentration. Originally, NO was described as an EDRF (Furchgott and Zawadski, 1980) that mediated the effects of ACh, for example (Ignarro et al., 1987; Palmer et al., 1987). It is now clear that neuronal NOS (Bredt et al., 1990; Bredt and Snyder, 1990, 1992) is a cytosolic enzyme that is abundant in the CNS whereby NO is produced postsynaptically in response to increase in intracellular Ca^{2+} upon, for example, glutamate receptor activation (Garthwaite, 1991). NO exerts its action via binding to the heme-Fe^{2+} prosthetic group of the soluble guanylyl cyclase, leading to activation and production of cyclic guanosine monophosphate (cGMP) (see Wolin et al., 1982).

In the autonomic nervous system, NOS is present in postganglionic cholinergic and probably noncholinergic parasympathetic nerves (Ceccatelli et al., 1992, 1994; Kummer et al., 1992) as well as in preganglionic cholinergic sympathetic nerves (Blottner and Baumgarten, 1992; Modin et al., 1994b). NOS has now been shown to be colocalized with VIP in presumably cholinergic parasympathetic postganglionic fibers, suggesting that these neurons can produce and release three principally different types of mediators: the classical transmitter ACh, peptides such as VIP, and the gas NO (Ceccatelli et al., 1992). Attenuation of NO production by NOS inhibitors, such as N^G-mono-methyl-L-arginine (L-NMMA), markedly reduces parasympathetic NANC relaxation of vascular (Garthwaite, 1991) and visceral smooth muscle (Gillespie et al., 1989, 1990; Andersson, 1993), suggesting that mechanisms involving NO are crucial for these events. Furthermore, mice carrying a selective mutation in neuronal NOS (NOS gene knockout mice) had grossly distended stomachs, possibly because of pyloric stenosis caused by lack of relaxant innervation (Huang et al., 1993).

C. Obstacles and Strategies for Cotransmission Studies

Concomitant release of multiple transmitters by transmural electrical field stimulation in in vitro preparations has been a common but sometimes ignored obstacle in demonstrating the involvement of various

and sneezing. It is obvious, however, that these sensory neurons also have important "motor functions" in the periphery due to local release of mediators (mainly peptides). Peptides are synthetized in sensory ganglion cell bodies, stored in LDV and then axonally transported (mainly in a peripheral direction). The mechanisms for peptide release from LDV involve increase in intracellular Ca^{2+}, either via receptor-operated channels or secondary to action potential generation and influx of Ca^{2+} through ω-conotoxin (CTX)-sensitive N-type Ca^{2+} channels (see lower magnified part). This type of action potential-evoked peptide release can be inhibited by TTX or a variety of prejunctionally active agents acting on μ-opioid receptors, α_2-adrenoceptors, Y_2-receptors or $GABA_B$-receptors, which all are likely to belong to the G-protein coupled family and may act via a common mechanism. Protons (H^+) and capsaicin seem to activate the same vanilloid receptor mechanisms, leading to influx of cations. The mechanisms for peptide release are different for the effects of low concentrations of protons and capsaicin (when they are similar to the effects of antidromic nerve stimulation and are dependent on axon reflexes) and for very high proton and capsaicin concentrations (when peptide release is TTX- and CTX-insensitive). Peripherally released glutamate (Glu) from small, clear vesicles may possibly activate KA receptors, causing influx of Ca^{2+}, although this action remains to be established. After release, peptides bind to specific receptors linked to various second-messenger systems (IP_3 for NK_1, NK_2 and NK_3 and cAMP and K_{ATP} channels for CGRP), finally evoking a variety of functional responses. These include plasma protein extravasation, leukocyte adhesion to endothelial cells, slow EPSP in autonomic ganglia (NK_1 in sympathetic and NK_3 in parasympathetic), contraction of smooth muscle in, for example, bronchi (NK_2 and NK_1) and ureter (NK_2), relaxation of smooth muscle in, for example, ureter (CGRP), vasodilation (CGRP) and stimulation of atrial contractility (CGRP). There are a variety of interactions between neuropeptides and inflammatory cell function, mediated via NK_1, NK_2 and CGRP receptors. Chronic activation of sensory nerves upon, for example, inflammation leads to an upregulation of peptide and receptor synthesis as well as sensory hyperalgesia due to changes in peripheral and central transmission mechanisms. ATP may be released from the cytoplasm of damaged cells into the extracellular space, subsequently activating P_{2X3} receptor subunits on capsaicin-sensitive sensory nerves (or in combination with P_{2X2}), leading to ion influx and depolarization.

mediators in autonomic neurotransmission. Thus, excitatory responses can be completely masked by the action of inhibitory transmitters and vice versa. Therefore, pretreatment with complex cocktails of antagonists has often been included as part of the experimental paradigm. It is also likely that in vitro conditions are not always representative of the in vivo situation in a variety of aspects, including basal nerve activity, basal release of NO from the endothelium caused by shear stress, presence or lack of diffusion barriers to mediators released from, for example, nerves at the adventitio-medial border and targeted to receptors on endothelial cells (especially in ring preparations) as well as degradation or binding of labile mediators such as NO to hemoglobin (Hb) in red blood cells. These complications are reduced in studies that use axonal stimulation of major nerves in vivo; however, even then, because of anatomical arrangements, sympathetic, parasympathetic and sensory fibers often are coactivated. Preganglionic and postganglionic axons or sensory nerves have different excitability, because of degree of myelinization, and the frequency dependency for mediator release varies. Except in sensory neurons (see Lundberg et al., 1994a), release of neuropeptides usually occurs mainly upon strong activation. Reflex stimulation is the preferred mode of selective activation of different pathways, but is sometimes difficult to combine with the anesthesia used, and sensory mediators released locally from the site of excitation can nonetheless complicate the interpretation of the data.

The major neuropeptides involved in cotransmission—SP, VIP and NPY—are members of families of closely related peptides. It is now recognized that so-called "specific" receptor antagonists often do not discriminate between receptors for different members of these peptide families because the peptides are full agonists at all receptor subtypes. Therefore, a major challenge is to determine the relative contribution of a number of closely related peptides to the cotransmission process. Biochemical identification of mediator forms that are released can assist in transmitter identification (even if degradation, or even bioactivation, occurs only after release). An alternative to receptor antagonism is an approach directed toward identifying the effects of the peptide. Initially, the actions of a particular peptide were blocked by immunoneutralization with specific antibodies; however, the antibodies are large molecules with limited penetration in tissues. In addition, knockout of peptide genes may be used in, for example, mice, although these changes may be lethal to the offspring or may lead to compensatory mechanisms. Nonetheless, this strategy to target the peptide or a specific enzyme, such as NOS, involved in transmitter synthesis may ultimately be necessary to elucidate the relative contribution of multiple peptides that use common receptor subtypes.

The release process for cotransmitters seems to be regulated by common prejunctional receptor mechanisms whereby endogenous NA or ACh, acting via prejunctional α_2 and m2 receptors, respectively, inhibit secretion of all mediators emanating from a varicosity. Therefore, antagonists or agonists at these receptors may have an especially marked effect on the nerve-evoked release of NANC mediators, such as neuropeptides and ATP, leading to changes in relative mediator contribution to the cotransmission process compared with normal conditions. Subtype-specific antagonists are becoming available for monoamines (Bylund et al., 1994) and should therefore be used instead of "general antagonists" such as phentolamine and atropine in future studies of cotransmission. It should also be emphasized that, because of restricted resupply by axonal transport, peptide release is more easily exhausted than (for example, classical transmitters) upon repeated or prolonged stimulations, which limits some experimental models.

Because many of the transmitters released interact (often with synergistic effects on the final effector response), this aspect must also be considered before the relative contribution of various transmitters to the autonomic neurotransmission process can be deduced from the actions of selective antagonists alone. Finally, aspects of development, age, species variations and changes under pathophysiological conditions or chronic drug treatment should be considered.

II. Neuropeptides, Glutamate and Sensory Nerves

A. Vanilloid Receptors

Capsaicin is uniquely selective for a subpopulation of sensory neurons, in which it causes dose-dependent excitation, desensitization and neurotoxicity. Absence of responses to tissue damage in capsaicin-treated animals is often interpreted as evidence that there is sensory efferent transmission from corresponding tissues in intact animals (Holzer, 1991). The selectivity of capsaicin can most likely be explained by a recently identified specific membrane recognition site, a "vanilloid receptor" (fig. 1). Direct evidence for this comes from membrane binding studies or autoradiography in which ^{3}H-resiniferatoxin (RTX) was used as the test ligand labeling a subset of sensory neurons (Szallasi and Blumberg, 1989, 1990; Szallasi et al., 1994) and from studies with the competitive vanilloid receptor antagonist capsazepine (Bevan et al., 1992; Szallasi et al., 1993). In addition, further support for the presence of such a specific recognition site is provided by (*a*) the existence of a structure-activity relationship for capsaicin-like activity (Szolcsanyi and Jansco-Gabor, 1975, 1976; Szallasi et al., 1989), (*b*) the remarkable cell selectivity of capsaicin for thin sensory neurons, (*c*) the ability of capsaicin to activate single-cation channels in mem-

brane patches from sensory neurons (Bevan and Szolcsanyi, 1990) and (*d*) the ability of nerve growth factor (NGF) to regulate the responsiveness to capsaicin at the cellular level (Winter et al., 1988).

Occupation of the specific recognition site (receptor) on the cell membrane of sensory neurons leads to opening of nonselective cation channels that are not affected by tetrodotoxin (TTX) or blockers of voltage-dependent Ca^{2+} channels, L-type such as nifedipine or N-type such as ω-conotoxin (CTX). Opening of these nonselective cation channels leads to influx of Na^+ and Ca^{2+}, causing depolarization (Wood et al., 1988; Bevan and Szolcsanyi, 1990), action potential generation and propagation of nerve impulses into peripheral ramifications and to the CNS, with subsequent transmitter release from peripheral and central branches of the sensory neurons (fig. 1). The local increase in intracellular Ca^{2+} concentration inhibits voltage-dependent Ca^{2+} channels. A high dose of capsaicin causes a rapid functional refractoriness, desensitization, followed by neurotoxicity caused by intracellular accumulation of Ca^{2+} and NaCl in small sensory neurons (B-type) in both spinal and visceral (such as nodose ganglion) sensory ganglia. Initially, Jancso et al. (1967) showed that rats treated with high doses of capsaicin after brief excitation not only became unresponsive to noxious chemical stimuli but also failed to develop neurogenic inflammation (plasma protein extravasation). This neurotoxic effect can be either slowly reversible (no somatic degeneration) or irreversible (degeneration of neuronal soma) and is associated with a delayed depletion of peptide transmitters (taking several hours). The acute defunctionalization upon capsaicin exposure is thus not caused by peptide depletion: this is a later event. Periaxonal or topical administration of capsaicin, unlike systemic treatment, has the advantage of producing an effective local ablation of sensory C-fiber neurons while exerting no systemic action (Holzer, 1991). Special attention must be given to age, strain and species differences regarding sensitivity to capsaicin and reversibility of its effects (Holzer, 1991). These differences can, at least partially, be related to heterogeneity of the vanilloid receptor system: there are differences not only between species, but also between central and peripheral branches of sensory neurons (Szallasi et al., 1993). Whether this indicates the presence of a family of endogenous ligands for the vanilloid receptors remains to be established.

TABLE 1
Regulatory mechanisms for sensory neuropeptide release

	Electrical nerve stimulation	Capsaicin		Low pH		Nicotine
		low	high	7 to 6	5	
Cation channel blockade (ruthenium red)	0	–	–	–	–	0
Vanilloid receptor antagonism (capsazepine)	0	–	–	–	(–)	0
NA^+-channel blockade (tetrodotoxin)	–	–	0	–	0	0
Ca^{2+} free medium (EGTA)	–	–	–	–	(–)	–
N-type Ca^{2+} channel blockade (omegaconotoxin)	–	–	0	–	0	0
α_2-receptor stimulation (UK14304)	–	–	0	–	0	0
Cyclooxygenase inhibition (indometacin)	0	0	0	–	0	(–)

Evoked by electrical nerve stimulation, capsaicin in low (10^{-8} M) or high (10^{-6} M) concentration, low pH (7 to 6 or 5) or nicotine (10^{-5} M). –, inhibition; 0, no effect; (–), partial inhibition.

B. Mechanisms and Regulation of Sensory Neuropeptide Release

1. Vanilloid receptors and low pH. Stored in LDV, sensory neuropeptides are released via exocytosis depending, for example, on influx of extracellular Ca^{2+} (Gamse et al., 1981). The dye ruthenium red selectively inhibits sensory neuropeptide release evoked by capsaicin, but not that evoked by nicotine or electrical stimulation (Maggi et al., 1988b, c; Franco-Cereceda et al., 1989b, 1991; Lou et al., 1991). Presumably, ruthenium red blocks the capsaicin-activated ion channels and, thereby, the influx of Ca^{2+} (Dray et al., 1990). Although it was originally postulated that capsaicin and electrical nerve stimulation cause neuropeptide release via two independent mechanisms (Maggi et al., 1988b, 1989b), subsequent studies showed that the mechanisms of capsaicin action are related to the concentration used. At a low concentration (10^{-8} M), capsaicin causes reproducible functional effects in isolated perfused organs, such as guinea pig lung, and causes neuropeptide release via TTX- and CTX-sensitive mechanisms, i.e., characteristics identical to those of responses to electrical antidromic vagal nerve stimulation (Kröll et al., 1990; Lou et al., 1991, 1992a) (table 1). At a high concentration in which tachyphylaxis to capsaicin is obtained, functional responses and peptide release are largely resistant to TTX and CTX, i.e., having characteristics similar to those of the responses seen after introduction of nicotine. Presumably, at low concentrations, capsaicin occupies only a proportion of the vanilloid receptors on sensory nerve endings. The elevated intracellular Ca^{2+} in nerve endings in which vanilloid receptors have been activated tends to inhibit voltage-dependent CTX-sensitive N-type Ca^{2+} channels in the same varicosities, but this local effect is minor compared with that evoked by the action potential propagation and peptide release at distant sites. Capsaicin at high concentrations causes major receptor occupancy and massive Ca^{2+} influx via receptor-operated channels, leading to pep-

tide release from most varicosities. At this stage, N-type Ca^{2+} channels are largely inhibited because of high intracellular Ca^{2+} concentrations, and the axon reflex mechanism for peptide release is of minor importance (Lou et al., 1992a). Capsazepine, the competitive antagonist of vanilloid receptors (Bevan et al., 1992; Szallasi et al., 1993), inhibits both capsaicin-evoked functional responses and peptide release from sensory neurons (Belvisi et al., 1992; Lou and Lundberg, 1992).

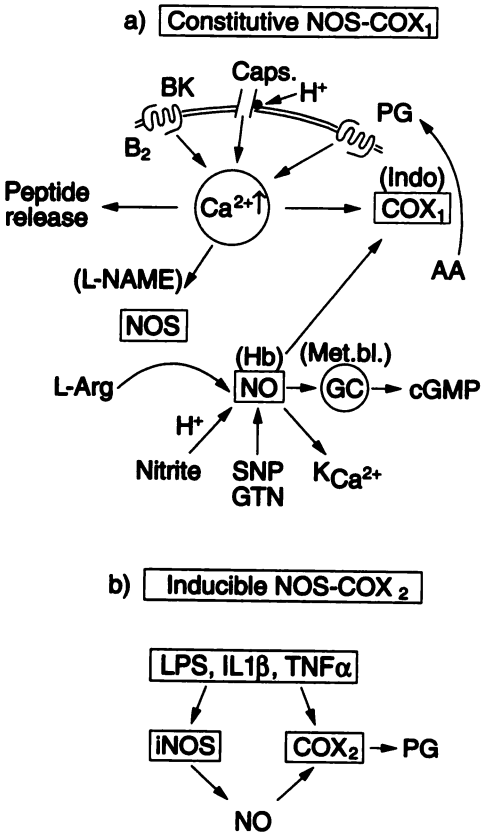

FIG. 2. (*a*) Model of various described alternatives involved in regulation of constitutive NOS and COX-1 activity by elevation in intracellular Ca^{2+}. Ca^{2+} increase is mediated either by receptor-operated channels, such as the capsaicin (Caps.) activation of the vanilloid receptor, which also seem to respond to protons (H^+), or by G-protein-coupled, receptor-mediated changes in IP_3, for example, via bradykinin (BK) acting on B_2 receptors. NO produced either endogenously by NOS from L-arginine and nonenzymatically by low pH from nitrite, or by NO donors such as sodium nitroprusside (SNP) or nitroglycerine (GTN) activates guanylyl cyclase (GC), leading to formation of cGMP. Furthermore, NO acts on COX-1, producing increased metabolism of AA and formation of various prostaglandins (PGs). In addition, NO itself can open Ca^{2+}-dependent K^+ channels, $K_{Ca}{}^{2+}$, apparently without requiring cGMP formation. PGs may modulate (sensitize) the vanilloid receptor and also further elevate intracellular Ca^{2+} by activating certain PG receptors. Sensory neuropeptide release can also represent a response to this Ca^{2+} change. It should be emphasized that NOS, COX and GC activation do not necessarily occur within the same cell because both NO and PGs can diffuse through membranes. For NO, this extracellular diffusion is neutralized by Hb. NOS is blocked by, for example, l-NAME, COX-1 by, for example, indomethacin (Indo) and guanylyl cyclase by, for example, methylene blue (Met.bl.). (*b*) Model of regulation of inducible nitric oxide synthase (i-NOS) and COX-2. The synthesis of these enzymes are induced by agents such as bacterial endotoxin (LPS), interleukin-1β and tumor necrosis factor α (TNFα). NO produced by a variety of sources (see *a*) can also increase the activity of COX-2, leading to exaggerated PG formation, at least when present in small amounts, whereas NO in high amounts may exert an inhibitory effet on COX-2 (see Swierkosz et al., 1995). Inducible NOS is inhibited both by L-NAME and more selectively by amino guanidine.

Low pH occurs in tissue upon ischemia, inflammation, intense muscle exercise or damage of the mucosal barrier of the stomach. Local pericellular pH may thus be sufficiently low near the region of sensory nerve endings to cause activation. Even under normal physiological conditions, pH is very low in the stomach (pH 2 to 3) and urine (pH 5). Disruption of mucosal and epithelial barriers may thus lead to acid diffusion into the tissue with stimulation of sensory neurons, leading both to local peptide release with various vascular and smooth muscle reactions and perception of pain and discomfort. It has been suggested that a "receptor" for protons is present in the membrane of sensory neurons, participating in, for example, nociception (Krishtal and Podiplicho, 1981). Functional studies suggest that gastric juice or lactic acid can evoke plasma protein extravasation in airway mucosa by causing local tachykinin release from sensory nerves (Martling and Lundberg, 1988; Auberson and Lundberg, 1993). Electrophysiological evidence suggests that protons (pH $<$ 6.2) open a cation channel similar to that opened by capsaicin (Bevan and Yeats, 1991; Liu and Simon, 1994). The opening of the channel by low pH can be sustained for minutes. Interestingly, the channel blocker ruthenium red inhibits acid-evoked (Forsberg et al., 1988; Liu and Simon, 1994) and SO_2-evoked (Aztori et al., 1992) activation of sensory nerves. Furthermore, the vanilloid receptor antagonist capsazepine inhibits low pH-evoked currents in trigeminal sensory neurons (Liu and Simon, 1994) and sensory neuropeptide release from the lung (Lou and Lundberg, 1992), skeletal muscle (Santicioli et al., 1993) and the heart (Franco-Cereceda et al., 1993). Also, in vivo, the reflex irritation (Lou and Lundberg, 1992), sensory bronchoconstriction (Satoh et al., 1993a, b) and nasal congestion (Rinder et al., 1994) responses to acid solutions are inhibited by capsazepine and/or capsaicin pretreatment. Finally, receptor binding data suggest that low pH modulates the RTX-sensitive vanilloid receptors (Szallasi et al., 1995). Taken together, these results indicate that low pH that occurs during inflammation and ischemia causes pain caused by activation of sensory nerves and that at least the final mechanisms in the activation are similar for low pH and capsaicin conditions, possibly involving a conformational change of the receptor or formation of some endogenous ligand(s) that interact with vanilloid receptors (figs. 1 and 2).

The mechanisms for peptide release by low pH seem to be dependent on the proton concentration, because the effect of moderately low pH (pH 7 to 6) is dependent on activation of TTX-sensitive Na^+ channels via N-type

Ca^{2+} channels, which are sensitive to CTX (Auberson and Lundberg, unpublished) causing influx of extracellular Ca^{2+} (Franco-Cereceda et al., 1994). Furthermore, the effect of lactic acid and moderately low pH on peptide release is inhibited by cyclo-oxygenase (COX) blockers, such as indomethacin and diclofenac (Franco-Cereceda et al., 1994). It is well known that prostaglandins stimulate ischemically sensitive afferents, and the response to ischemia is attenuated by COX blockade (Longhurst and Dittman, 1987; Longhurst et al., 1991). In contrast, at pH 5, peptide release seems to occur largely independently of influx of extracellular Ca^{2+} or prostaglandin formation (Lou and Lundberg, 1992; Bevan and Geppetti, 1994; Franco-Cereceda et al., 1994) (table 1). Certain prostaglandins (PGs) such as prostacyclin (PGI_2) are formed upon low pH and can release peptides from sensory nerves; interestingly, the peptide release, but not the formation of PGI_2 upon low pH in the heart, can be attenuated by capsazepine (Franco-Cereceda et al., 1994). Furthermore, PGI_2 activates tachykinin (TK) release from capsaicin-sensitive afferents in guinea pig bronchi through a ruthenium red-sensitive pathway (Mapp et al., 1991). In analogy with the similarity to electrical nerve stimulation and low concentration of capsaicin, moderately low pH (pH 6) evokes peptide release that is inhibited by an α_2-agonist (Auberson and Lundberg, unpublished data) (table 1). Overall, these findings are compatible with the idea that protons and capsaicin activate sensory neurons by a common mechanism that is sensitive to ruthenium red and capsazepine and presumably involves the vanilloid receptor complex (figs. 1 and 2). Another possible mechanism involved in the sensory nerve activation by low pH could be a nonenzymatic formation of NO (Lundberg et al., 1994b) with possible COX activation and PG formation (fig. 2).

2. Electrical nerve stimulation and multiple irritants. In contrast to overflow of other neuropeptides such as VIP (Lundberg, 1981) and NPY (Lundberg et al., 1990), even a low electrical stimulation frequency seems to be optimal for release of sensory neuropeptides (Lou, 1993; Lundberg et al., 1994a)—even though capsaicin-sensitive C-fiber afferents are capable of transmitting brief bursts of action potentials at relatively high frequency (Coleridge and Coleridge, 1984; Lee et al., 1989). Nerve conduction block seems to develop rapidly in C-fiber afferents upon high stimulation frequencies, however (Torebjörk and Hallin, 1974). Even single impulse stimulation of sensory nerves can cause antidromic vasodilation in the skin (Pierau and Szolcsanyi, 1989), and bronchoconstriction (Lou, 1993) and low frequencies (a few Hz) produced maximal vasodilator or bronchoconstrictor effect (Celander and Folkow, 1953; Lindén et al., 1991; Lou, 1993) and hyperpolarization of smooth muscle in the ureter (Santicioli and Maggi, 1994).

Bradykinin formed upon plasma protein leakage and histamine released from mast cells can stimulate C-fiber afferents and trigger secretion of sensory neuropeptides, presumably via B_2 (Dray and Perkins, 1988; Franco-Cereceda et al., 1989a) and H_1 receptors, respectively (Saria et al., 1988; Alving et al., 1990) (fig. 1). The bradykinin effect is also sensitive to CTX (Gepetti et al., 1990). Finally, COX inhibition can reduce peptide release by bradykinin, again suggesting an important relationship between prostanoids and sensory neuropeptide release (fig. 2) (Geppetti et al., 1991; Hua et al., 1994). The classical axon reflex flare reaction to allergen in the human skin seems to involve both histamine and COX products, considering the inhibitory effects of H_1 and COX antagonists (Fuller et al., 1987; Wallengren and Håkanson, 1987; Wallengren, 1991). Nicotine can activate capsaicin-sensitive C-fiber afferents (Lundberg et al., 1983b; Lee et al., 1989), presumably via N2-nicotinic receptors (Kizawa and Takayanagi, 1985; Saria et al. 1988; Lou et al., 1992a). The nicotine effect does not involve axonal reflexes or N-type Ca^{2+} channels to any major extent (Lou et al., 1992a). Presumably, nicotine evokes Ca^{2+} influx directly through receptor-operated channels (Noronka-Blob et al., 1989) (fig. 1). Recently, it has been reported that the nicotine effect to some extent also involves activation of COX (Hua et al., 1994). Also, members other than PGs of the arachidonic acid cascade can activate or sensitize sensory nerve endings (Manzini et al., 1989). Thus, lipoxin A_4 causes bronchoconstriction in the guinea pig via a partially capsaicin-sensitive sensory pathway (Manzini and Meini, 1991). Furthermore, leukotriene D_4 and platelet activating factor can also release sensory neuropeptides from the lung (Martins et al., 1991), and leukotriene antagonists have been reported to interfere with the vagally evoked sensory bronchoconstriction (Ellis and Undem, 1991).

Inhibition of K^+ channels using 4-amino-pyridine (Rudy, 1988) enhances stimulation-evoked transmitter release from a variety of neurons. This also occurs in capsaicin-sensitive sensory nerves, inasmuch as the peptide overflow evoked by antidromic vagal stimulation is enhanced by 4-amino pyridine (Lou and Lundberg, 1993). Furthermore, 4-amino pyridine per se seems to induce action potentials (Stansfeld et al., 1986) and peptide release from vagal afferents (Lou and Lundberg, 1993).

Experimental evidence also suggests that nitrovasodilators, such as sodium nitroprusside, activate nociceptive fibers to release CGRP that may contribute to the vasodilatory effects of NO donors, both in the cerebral circulation (Wei et al., 1992) and skin (Holzer and Jocic, 1994). Biochemical evidence suggests that NO can interact directly with COX to cause an increased formation of prostaglandins (Salvemini et al., 1993) (fig. 2). Recent data, in fact, suggest that sodium nitroprusside-evoked skin vasodilation caused by CGRP release involves COX products (Holzer et al., 1995).

ATP is known to depolarize sensory neurons and may play a role in nociceptor activation when released from damaged cells (fig. 1). Recently, several subtypes of P_{2X}

receptors have been cloned and found to constitute a family of cation channels (see Kennedy and Leff, 1995a, b; Surprenant et al., 1995). Messenger RNA for the P_{2X3} receptor has been found exclusively in small-diameter, capsaicin-sensitive neurons, specifically dorsal root and trigeminal ganglia (Chen et al., 1995a; Lewis et al., 1995). This subunit can form functional channels when expressed on its own and can also form a heteromultimer with the P_{2X2} subunit, which normally has a wider distribution. The P_{2X3} seems to determine the receptor's pharmacological properties, whereas P_{2X2} controls the kinetics of the current. The present example with P_{2X} receptors on sensory neurons in which subunits can assemble as either homopolymers or heteropolymers has clear parallels to nicotinic and glutamate receptor channels (see Lewis et al., 1995). The highly selective localization of P_{2X3} on nociceptor afferents suggests future therapeutic possibilities for the development of novel analgesics. The possible relationship between this receptor and the not yet cloned vanilloid receptor(s) also remains to be determined.

3. Prejunctional inhibition. Peptide release from sensory nerves can be inhibited prejunctionally by a variety of agents (see Barnes et al., 1990), including α_2-adrenoceptor agonists, such as UK14304 or opiate receptor agonists (Bartho et al., 1987; Matran et al., 1989b) (fig. 1). This inhibitory effect seems to be most prominent on the response to low capsacin concentration, antidromic nerve stimulation, moderately low pH (table 1) and bradykinin (Lou et al., 1992c), whereas the effect of nicotine is uninfluenced (Lou et al., 1992a). In addition to prejunctional α_2-adrenoceptor and opiate receptor regulation, NPY receptors (Matran et al., 1989b) of the Y_2 type (Grundemar et al., 1990b) and $GABA_B$ receptors (Ray et al., 1991; Bowery, 1993) can inhibit peptide release from sensory nerves. Presumably, the prejunctional α_2-mechanisms depend on regulation of Ca^{2+} influx through N-type voltage-gated channels (Stjärne, 1989). It has also been suggested that high conductance Ca^{2+}-activated K^+ channels play a role in μ-opioid receptor-induced inhibition of peptide release from airway sensory nerves (Stretton et al., 1992). It is of interest that morphine, for example, inhibits antidromic vasodilation, presumably because of interference with neuropeptide release, without affecting the excitability of C-polymodal nociceptors in the skin. This suggests that propagation of the nerve impulse is not affected to the same extent as Ca^{2+} influx necessary for peptide release (Shakhanbeh and Lynn, 1993). Several compounds known to elevate cellular cAMP inhibit sensory bronchoconstriction, apparently without influencing postjunctional responses (Aikawa et al., 1992). Furthermore, inhibition of phosphodiesterase (PDE) IV, which also elevates cAMP, can attenuate functional responses caused by peptide release from airway sensory nerves, although this should be confirmed using biochemical measurements (Undem et al., 1994; Qian et al., 1994). Interestingly, histamine 3 receptors inhibit peptide release from sensory nerves (Ohkubo et al., 1995), suggesting that this autacoid has a complex action, inasmuch as

TABLE 2
Transmitter characteristics for sensory neuropeptides, NKB and glutamate

	SP	NKA	NKB	CGRP	Glutamate		
Synthesis	sensory cell body PPT I precursor	sensory cell body PPT I precursor	CNS cell body PPT II precursor	sensory cell body precursor	glutamine cycle		
Storage	LDV	LDV	–	LDV	small vesicles		
Terminal resupply	axonal transport	axonal transport	–	axonal transport	reuptake, synthesis		
Preferred receptor	NK_1	NK_2	NK_3	$CGRP_{(1,2)}$	AMPA	NMDA	KA
Selective agonist	Sar9($(MetO_2)^{11}$)SP	(β-ala^8]NKA(4–10)	senktide (NKA>>SP)	CGRP	AMPA	NMDA	KA
Antagonist	RP67580 (rat) CP96345 (human) SR 140333	SR48968	SR142801	CGRP(8–37)	NBQX	AP5	NS102
Second-messenger	IP_3, Ca^{2+}, PKC	IP_3, Ca^{2+}, PKC	IP_3, Ca^{2+}, PKC	cAMP, K_{ATP}	Na^+, K^+ (fast)	Na^+, Ca^{2+} (slow) NO	Na^+ K^+ (fast)
Functional response	plasma protein leak very slow EPSP vasodilation smooth muscle contraction exocrine secretion	smooth muscle contraction	ACh release very slow EPSP	vasodilation tachycardia smooth muscle relaxation	fast EPSP	slow EPSP LTP	fast EPSP
Removal	NEP, ACE	NEP	–	(NEP)	reuptake		

Summary of transmitter characteristics for sensory neuropeptides and glutamate, which coexist in capsaicin-sensitive neurons and NKB.

PPT, preprotachykinin; LDV, large dense-cored vesicles; NK, neurokinin; IP_3, inositoltrisphosphate; PKC, protein kinase C; K_{ATP}, ATP-sensitive K^+ channel; PKC, protein kinase C; LTP, long-term potentiation; NEP, neutral endopeptidase; ACE, angiotensin converting enzyme.

For NKB, only synthesis, receptor characteristics and functional response has been included because this peptide is not found in sensory neurons. The NK_3 receptor is preferentially activated by other TKs, presumably NKA released from sensory nerves.

H1 receptor activation stimulates peptide release (Saria et al., 1988). Because multiple neuropeptides are released upon activation of sensory nerves, a prejunctional inhibition of release may cause a more efficient blockade of functional responses than receptor antagonists to single neuropeptides (fig. 1).

C. Tachykinins as Neurotransmitters

1. Synthesis, release and degradation. TKs represent a family of peptides, and the name comes from their rapid initiation of smooth muscle contraction (compared with the slower-acting bradykinin) (Pernow, 1983). TKs possess a common carboxyl terminus Phe-X-Gly-Leu-Met-NH_2 in which X represents Phe or Val in mammalian forms. The C-terminal amide is essential for biological activity. SP was initially isolated as a crude extract from equine brain and gut and was found to have potent hypotensive and smooth muscle contractile properties (von Euler and Gaddum, 1931). This compound was named *substance P*, in which P stands for the powder obtained after the extraction procedure. SP was sequenced by Chang et al. (1971). Two novel decapeptides similar to SP, now referred to as neurokinin A (NKA) and neurokinin B (NKB), were subsequently isolated from CNS (Kangawa et al., 1983; Kimura et al., 1983). Alternative splicing of mammalian tachykinin mRNAs, followed by differential posttranscriptional processing can give rise to N-terminally extended forms of NKA. Neuropeptide K (NPK, with 36-amino-acid residues) was isolated from porcine brain (Tatemoto et al., 1985) and demonstrated in peripheral sensory neurons (Hua et al., 1985). Neuropeptide γ (NPγ, a 21-amino-acid peptide) was subsequently discovered in rabbit intestine (Kage et al., 1988). Shorter forms may also exist because immunoreactivity to NKA(3–10) has also been reported in mammalian tissue extracts (Theodorsson-Norheim et al., 1987).

TKs are produced by ribosomes within the neuronal cell body, packed into storage vesicles (of the LDV type) (Merighi et al., 1988; Plenderleith et al., 1990) and axonally transported to terminal endings for final enzymatic processing (Brimijoin et al., 1980; Maggio, 1988; Munekata, 1991) (table 2). Sensory neurons express mainly the preprotachykinin I (PPT I) gene, producing SP, NKA and the other NKA-related peptides (Too et al., 1989a, b; Takeda et al., 1990; Helke et al., 1990) (table 1). The PPT II gene, which encodes NKB, is expressed in the CNS but not in sensory neurons; hence, NKB is absent from peripheral terminals of capsaicin-sensitive afferents (Hua et al., 1985; Too et al., 1989a, b) (table 2). TKs synthesized by sensory ganglion cells are transported in both central and peripheral directions, and their release is involved both in the afferent and efferent functions of the capsaicin-sensitive sensory neurons. In this context, it is of interest that 90% of the TKs produced by sensory cells in the vagal system seem to be transported out into the peripheral endings, suggesting that, quantitatively, the efferent functions of these peptides may be most relevant (Brimijoin et al., 1980) (fig. 1). Principally similar findings have also been reported for spinal ganglion cells (Harmar and Keen, 1982). Production of TKs in sensory nerves can be influenced by pathophysiological stimuli, such as inflammation, when it is increased (Noguchi et al., 1988), or peripheral axotomy, when SP production is reduced (Jessell et al., 1979; see Hökfelt et al., 1994).

SP and NKA release are dependent on an influx of extracellular Ca^{2+} in response to depolarizing stimuli (Saria et al., 1983a, 1986, 1988; Maggi et al., 1988a, b). The simultaneous release of SP and NKA from sensory neurons has also been demonstrated in a variety of peripheral organs following application of capsaicin and various inflammatory agents (bradykinin, histamine) known to be produced during tissue damage and inflammation (Hua et al., 1986; Saria et al., 1988; Geppetti et al., 1990, 1991; Lou, 1993). It has also been proposed that the N-terminally extended forms of NKA, i.e., NPK and NPγ, or of SP (Toresson et al., 1990a), act as transmitters (Tatemoto et al., 1985; Dam et al., 1990). NPK has been identified in human cerebrospinal fluid (Toresson et al., 1990b), but Hua et al. (1985) failed to detect release of NPK from sensory nerves in the lung, although tissue extracts contained measurable amounts of the peptide. Martling et al. (1987) could, however, show that extraneuronal cleavage of NPK to NKA can occur in the guinea pig in vivo. In summary, firm evidence for a release of the N-terminally extended forms of NKA is not yet available. Because TKs are rapidly degraded, experiments using peptidase inhibitors may be required to demonstrate this, especially in guinea pig lung (Lou, 1993). Also, other fragments of NKA, such as NKA(3–10), may be released from sensory nerves (Hua et al., 1985; Theodorsson-Norheim et al., 1987), although this remains to be conclusively demonstrated. The existence of several NK receptor subtypes may, however, imply that many different TK ligands are released.

TKs are highly susceptible to hydrolysis by tissue-specific enzymes, which can be cytosolic, or membrane-bound. Neuronal reuptake appears to play no role in terminating the action of TKs and to act by removing degradation fragments rather than the intact peptide (Nakata et al., 1981). When biological effects of TKs are tested, it is important to consider that the various peptides are not equally susceptible to enzymatic degradation. Hence, many experiments are conducted routinely in the presence of peptidase inhibitors, although this may also change the relative contribution of different TKs to the functional responses. Neutral endopeptidase-24.11 (NEP) (EC.3.4.24.11) is found in the lining epithelium and within smooth muscle in peripheral tissues, such as airways; in these tissues, it is present in large quantities (Sekizawa et al., 1987). NEP rapidly hydrolyzes three bonds within the backbone and C-terminus of SP (Matsas et al., 1984; Skidgel et al., 1984; Martins

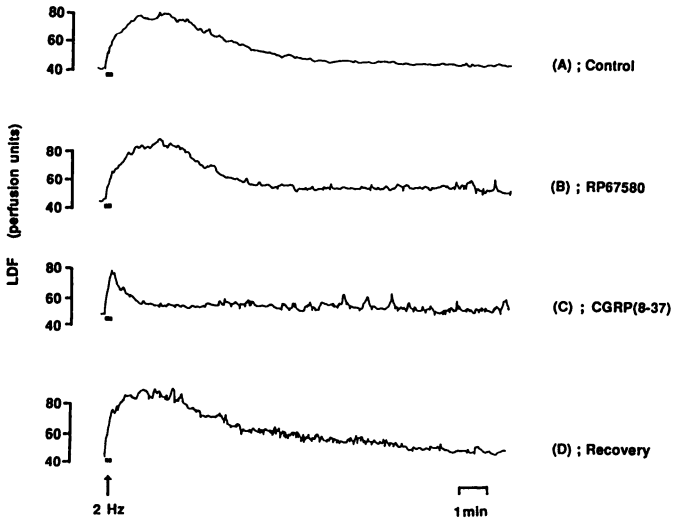

FIG. 3. Effects of the nonpeptide NK_1 antagonist RP67580 (1 mg/kg i.v.) and the $CGRP_1$ antagonist CGRP(8–37) (0.3 mg/kg i.v.) on the vasodilation response (expressed in Laser Doppler flow meter, LDF perfusion units) in dorsal skin of rat hindpaw in vivo upon electrical antidromic stimulation of capsaicin-sensitive nerves in the saphenous nerve with 20 impulses (2 Hz for 10 seconds, bar) at 30-minute intervals (*A*). Pretreatment with guanethidine (5 mg/kg, 24 h earlier) was used to inhibit the effects of coactivation of sympathetic fibers (modified from Delay-Goyet et al., 1991). Note that the brief stimulation causes a very long-lasting response. The NK_1 antagonist is without effect (*B*), while CGRP(8–37) markedly (*C*) and reversibly (*D*) reduces especially the long-lasting component of the response. Bar indicates 1 minute.

et al., 1990). NEP also cleaves the Gly^8-Leu^9 bond in NKA (table 2). Phosphoramidon and thiorphan are selective NEP inhibitors, which markedly potentiate responses evoked by exogenous and endogenously released TKs (fig. 4) (Matsas et al., 1984; Sekizawa et al., 1987; Dusser et al., 1988; Thompson and Sheppard, 1988) as well as increase TK outflow into the venous effluent of isolated organs after release (Kröll et al., 1990; Lou, 1993). Thiorphan also enhances airway responses to NKA in normal humans in vivo (Cheung et al., 1992). It should be emphasized that NEP is involved in degradation of a variety of peptides including CGRP, VIP and NPY. Angiotensin-converting enzyme (ACE) (EC 3.4.15.1) can also hydrolyze C-terminal bonds of SP (Yokosawa et al., 1983; Skidgel et al., 1984). In contrast to NEP, ACE occurs mainly in endothelial cells of the respiratory tract so that treatment with ACE inhibitors (e.g., captopril) potentiates the responses only when agonists are administered through vascular routes (Drazen et al., 1989; Martins et al., 1990). NKA is not influenced by ACE: this probably explains why NKA has a much longer half-life when given i.v. than does SP (Martling et al., 1987). Dipeptidylaminopeptidase IV (EC 3.4.14.5) also seems to degrade both SP (Heymann and Mentlein, 1978), NKA (Nau et al., 1986) and other neuropeptides.

2. Biological actions of tachykinins. Three receptor types—termed neurokinin 1 (NK_1), NK_2, and NK_3—mediate the biological effects of TKs. The main second-messenger system coupled to activation of the three known receptor types is stimulation of phospholipase C, leading to phosphoinositide breakdown (Guard and Watson, 1991). Bioassay in vitro, radioligand binding and in vivo physiological experiments have led to the recognition of NK_1, NK_2 and NK_3 receptors (Hua et al., 1984; Nawa et al., 1984; Lee et al., 1986; Maggi et al., 1987c; Regoli et al., 1987). Molecular biology studies have confirmed this characterization, and three distinct genes encoding three NK receptor proteins have now been cloned (Nakanishi, 1991). Natural TKs bind to these receptors with a certain amount of selectivity: NK_1 receptors prefer SP, NK_2 receptors NKA, and NK_3 receptors NKB (table 2). Nonetheless, these peptides can act as full agonists at all three receptor types, albeit with different affinities (Burcher et al., 1980; Regoli et al., 1987; Nakajima et al., 1992), Thus, it is likely that there is extensive binding promiscuity between coreleased endogenous TKs and different NK receptor subtypes (see Burcher et al., 1991). A major consideration in studies on TK receptors concerns metabolism of endogenous peptides by peptidases, which can result in inappropriate classification of the TKs involved in given response. Synthetic, metabolically stable receptor-selective agonists are useful in this respect, and the recently developed nonpeptide TK receptor antagonists have provided further evidence for the existence of NK receptor subtypes (see Regoli et al., 1994).

The wide distribution of TK-containing sensory nerves in the periphery is matched by a variety of biological effects that occur in most mammalian species (Pernow, 1983). Some of these well known actions are presented in the next eight paragraphs.

a. VASODILATION. Vasodilation is measurable as transient hypotension or increase in local arterial blood flow following intravascular administration of TKs in vivo, or relaxation of isolated precontracted blood vessels in vitro (Pernow, 1983). The effect is mediated by NK_1 receptors (Hua et al., 1984; Regoli et al., 1987; Couture et al., 1989; Delay-Goyet et al., 1992b, 1993) located on endothelial cells (Stephenson et al., 1986) and involves the release of NO in at least some vascular beds (Whittle et al., 1989; Persson et al., 1991), although SP can clearly evoke vasodilation, even after inhibition of NOS (Kerezoudis et al., 1993a). It is not clear whether the endothelial NK_1 receptors are "innervated" in the sense that TKs released from the often-abundant perivascular nerves on the adventitial side (Gulbenkian et al., 1986) can penetrate the vascular wall (fig. 1). Thus, relaxation of isolated blood vessels caused by sensory nerve activation by capsaicin or electrical field stimulation is not changed by endothelium removal (Franco-Cereceda et al., 1987a; Maggi et al., 1990b; Li and Duckles, 1992). Furthermore, sensory nerve-evoked vasodilation, at least in the skin, in vivo is largely unchanged by specific NK_1 receptor antagonists (fig. 3), whereas the effect of exogenous SP is abolished (Delay-Goyet et al., 1992b,

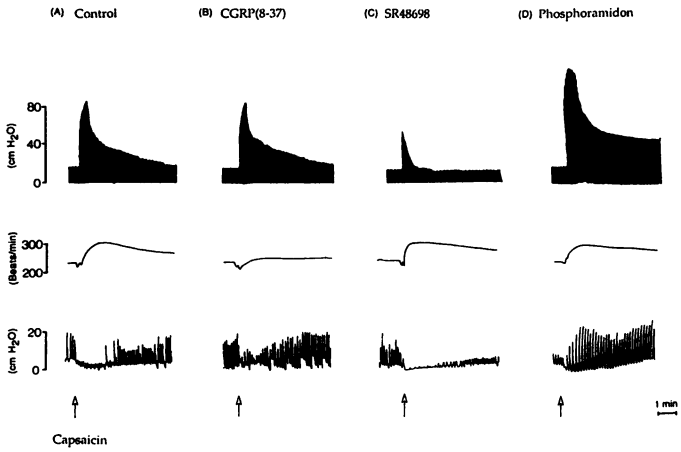

FIG. 4. Effects of i.v. capsaicin (30 μg/kg) in anesthetized guinea pigs pretreated with the ganglionic N_2 nicotinic receptor blocker chlorisondamine to inhibit autonomic reflexes, thereby revealing local sensory motor function using multiple peptide mediators affecting bronchomotor tone (*upper panel*) (recorded by insufflation pressure), heart rate (*middle panel*) and ureter motility after obstruction (*lower panel*) in a control animal (*A*), after the $CGRP_1$ receptor antagonist CGRP(8–37) (0.3 mg/kg i.v.) (*B*), after the nonpeptide NK_2 antagonist SR48968 (0.3 mg/kg i.v.) (*C*) or (*D*) after the NEP inhibitor phosphoramidon (5 mg/kg i.v.). Note that CGRP(8–37) attenuates both tachycardia and the inhibition of ureter motility, whereas the NK_2 antagonist SR48968 attenuates bronchoconstriction and augments the inhibitory response in the ureter. Phosphoramidon enhances bronchoconstriction and ureter contractions; and this latter result suggests that NEP is more important for TK (excitatory transmitter) than CGRP (inhibitory transmitter) degradation. Time scale is 1 minute.

1993; Kerezoudis et al., 1994; Rinder and Lundberg, 1995). In certain blood vessels, a *vasoconstrictor* response to TKs is observed; this is mediated by NK_1 receptors in rabbit jugular vein (Nantel et al., 1990), NK_2 receptors in rabbit pulmonary artery (D'Orléans-Juste et al., 1986) and NK_3 receptors in rat portal vein and mesenteric circulation (Dion et al., 1987; Mastrangelo et al., 1987; D'Orléans-Juste et al., 1991).

b. VISCERAL SMOOTH MUSCLE CONTRACTION. Visceral smooth muscle contraction is mostly a direct effect of TKs on smooth muscle cells and supports the role of TKs as excitatory transmitters released from locally activated sensory nerves in bronchial smooth muscle (fig. 4) (Lundberg et al., 1983a; Lundberg and Saria, 1987), intestine (Bartho and Holzer, 1985) and urinary tract (Maggi et al., 1991a). One factor that complicates interpretation of the contractile effects of TKs is the fact that preparations often contain both NK_1 and NK_2 receptors (e.g., guinea pig bronchus) or even all three NK receptors (e.g., guinea pig ileum) (Dion et al., 1987). Furthermore, TKs can cause bronchodilation in the mouse because of release of COX products from airway epithelial cells (Manzini, 1992).

c. PLASMA PROTEIN EXTRAVASATION. Plasma protein extravasation by TKs is highly localized to mucosal membranes and skin (Saria et al., 1983b; Lundberg et al., 1983d, 1984b), where TKs lead to leakage of plasma proteins from postcapillary venules (fig. 1). This reaction is especially prominent in rat and guinea pig and is considerably slower and needs much stronger stimuli than the vasodilation in skin or airway mucosa seen upon antidromic stimulation of sensory nerves. It is perhaps significant that the nerve profiles are mostly much farther away from postcapillary venules than from arterioles (McDonald et al., 1988). Therefore, relatively large amounts of TKs may have to be released and then possibly even resorbed into the capillary circulation before activating NK_1 receptors on the endothelium of postcapillary venules. The identification of the NK_1 receptor as the entity that mediates sensory neurogenic plasma protein extravasation has been substantiated in experiments using both endogenous TKs (Hua et al., 1984), selective agonists (Abelli et al., 1991) and antagonists (Delay-Goyet and Lundberg, 1991a; Delay-Goyet et al., 1992a, 1993) (fig. 1). This leakage is normally transient and undergoes rapid desensitization. Internalization of NK_1 receptors by endothelial cells may be one of the mechanisms that limit the amount of plasma leakage at sites of inflammation (Bowden et al., 1994). Additional data suggest that in peripheral bronchi of the guinea pig also, NK_2 antagonists attenuate the plasma extravasation response (Tousignant et al., 1993).

d. SLOW EXCITATORY TRANSMISSION FROM SENSORY COLLATERALS IN AUTONOMIC GANGLIA. Distension of the ureter (Amann et al., 1988) or the colon (Kreulen and Peters, 1986; Hankins and Dray, 1988) evokes a slow EPSP of sympathetic ganglion cells via a sensory transmitter that facilitates the cholinergic ganglionic transmission; this serves as a short-loop reflex control of visceral activities. Several lines of evidence suggest that certain tachykinins, i.e., SP, serve as neurotransmitters for slow EPSPs in mammalian prevertebral postganglionic sympathetic ganglion cells evoked by e.g., capsaicin (Konishi et al., 1979, 1992; Konishi and Otsuka, 1985). Because not only SP but also NKA evokes similar depolarization (Saria et al., 1987), several neurokinin receptor subtypes could be involved in the synaptic transmission (fig. 1). Subsequent pharmacological characterization revealed that both NK_1 and NK_3 agonists evoked depolarization in guinea pig sympathetic neurons, and the NKA-induced response was antagonized by an NK_1 antagonist (Zhao et al., 1993). The slow depolarization in sympathetic ganglion cells evoked by electrical stimulation of sensory nerves was also inhibited by an NK_1 antagonist, suggesting major involvement of NK_1 receptors as the final effector for endogenously released TKs (Zhao et al., 1993). Facilatory mechanisms by sensory TKs may also be present in parasympathetic ganglia of the airways, leading to enhanced ACh release from parasympathetic nerves (Lundberg et al., 1983d; Martling et al., 1984; Undem et al., 1990; Watson et al., 1993). In the parasympathetic bronchial ganglia, however, TKs mediate their effects primarily via NK_3 receptors as revealed by desensitization experiments using the NK_3 agonist

senktide and the NK_1 antagonist CP96345 (Myers and Undem, 1993).

e. STIMULATION OF EXOCRINE SECRETION. Stimulation of exocrine secretion by TKs is evident in, for example, salivary glands. This effect is, however, very species-dependent; although it is prominent in the rat, it cannot be found in salivary glands of cat or humans (Larsson et al., 1986). In the rat, salivary glands NK_1 receptors are involved in the secretory response (Giuliani et al., 1988; Snider et al., 1991), and TKs have been implied as mediators of atropine-resistant salivary secretion upon electrical stimulation of the parasympathetic nerve supply; however, this may not be relevant for sensory function because SP is also present in parasympathetic neurons in local ganglia of salivary glands in this species (Ekström et al., 1988). TKs also stimulate secretion from airway glands (Rogers et al., 1989; Gentry, 1991) via an NK_1 mechanism (Geppetti et al., 1992). Finally, NK_1 receptors mediate the increase in mucociliary activity in airway mucosa produced by tachykinins (Lindberg and Dolata, 1993).

f. RECRUITMENT OR STIMULATION OF INFLAMMATORY CELLS. Recruitment or stimulation of inflammatory cells is a proinflammatory action of TKs. Thus, TKs, like SP, can evoke adhesion of neutrophils and eosinophils to endothelial cells via expression of an endothelial-leukocyte adhesion molecule (Matis et al., 1990) that is the first step in the migration of leukocytes into inflamed tissues (McDonald, 1988; Baluk et al., 1995). Apparently, neutrophil adhesion has a lower threshold of sensory nerve activation than plasma protein extravasation (Katayama et al., 1993) (fig. 1), although both reactions may depend on the fact that neuronally released TKs from nerves around arteries are resorbed into the circulation via capillaries, subsequently activating endothelial NK_1 receptors. TKs can also enhance lymphocyte proliferation (Payan et al., 1983, 1984), stimulate alveolar macrophages (Brunelleschi et al., 1990, 1992), neutrophils (Brunelleschi et al., 1991), eosinophils (Kroegel et al., 1990) and monocytes (Wagner et al., 1987; Lotz et al., 1988) and activate rheumatoid synoviocytes (Lotz et al., 1987). Both NK_1 and NK_2 receptors can be involved in these effects. Furthermore, SP has been reported to enhance secretion of tumor necrosis factor-α from neuroglial cells (Luber-Narod et al., 1994).

g. MAST CELL DEGRANULATION. Mast cell degranulation seen, for example, in human skin upon local SP injection (Hägermark et al., 1978) is caused by the basic N-terminal sequence of SP, possibly through a direct activation of G-proteins on the mast cell membrane (Mousli et al., 1990). Even if TK-containing sensory nerves can be seen close to mast cells, suggesting a functional interaction (Nilsson et al., 1990; Alving et al., 1991), the relevance of sensory neurogenic control of mast cell function is not clear (Saria et al., 1984). Possibly, rather than being a direct degranulating agent, endogenous SP may prime mast cells to react to other agents released from sensory nerves (Janiszewski et al., 1992). It is of interest that skin reactivity to SP in humans is increased in allergic asthmatics (Iwamoto et al., 1990). As discussed above, there is ample evidence that mediators—including histamine—released from mast cells can activate sensory nerves with subsequent action potential propagation and peptide release, causing the flare response upon allergen challenge (fig. 1) (Alving et al., 1990), although TKs may not be of primary importance in the vasodilation part of this response.

h. TROPHIC ACTIONS OF TACHYKININS. Trophic actions of TKs released by noxious or harmful stimuli enhance the inflammatory response (by vascular events and by influencing inflammatory cells). It has been postulated that sensory neuropeptides also play a key role in long-term maintenance of normal trophism of the skin and cornea, because cutaneous and corneal lesions are induced by neonatal systemic capsaicin treatment (Maggi et al., 1987a; Szolcsanyi and Bartho, 1981; Abelli et al., 1993). Among the obvious trophic roles of TKs are protection of the tissue from acute injury and promotion of tissue repair (Holzer, 1988). It is also clear that capsaicin-sensitive sensory nerves are of importance in the maintenance of tissue integrity in the gastric mucosa (Lippe et al., 1989; Holzer, 1991). TKs exert powerful mitogenic or permissive actions on growth of various cell types such as fibroblasts (Nilsson et al., 1985; Ziche et al., 1990a; Harrison et al., 1992), synoviocytes (Lotz et al., 1987), endothelial cells (Ziche et al., 1990b), and macrophages (Brunelleschi et al., 1990). TKs stimulate not only endothelial cell proliferation but also migration and angiogenesis (Ziche et al., 1990b, 1991) that could be of relevance during tissue growth and repair.

3. Neurokinin receptors. a. NK_1 RECEPTORS. Pharmacologically characterized NK_1 receptors are abundant in visceral smooth muscle, endothelial cells and exocrine glands. Classic examples are found in the respiratory, gastrointestinal, and urogenital tracts. The vasodilation and plasma protein extravasation evoked by SP are mediated by NK_1 receptors. A 407-amino-acid residue G-protein-coupled receptor protein has been cloned, at which SP is a better ligand than NKA or NKB (Yokota et al., 1989; Hershey and Krause, 1990; Gerard et al., 1991). The rapid desensitization of many NK_1 receptor responses may be related to phosphorylation in the C-terminal cytoplasmic region of the receptor (Hershey and Krause, 1990) and/or receptor internalization (Bowden et al., 1994). The new nonpeptide NK_1 competitive receptor antagonists CP96345 (Snider et al., 1991) and RP67580 (Garret et al., 1991) are very potent (nM range) but show distinct species-related differences in antagonistic activity. Thus, at human, guinea pig, and rabbit NK_1 receptors, CP96345 is much more potent, whereas in the rat or mouse, RP67580 is the most potent (Fardin et al., 1992). It is possibe that minor changes in transmembrane segments of the NK_1 receptor are responsible for the observed differences in antagonist (not agonist)

affinities between the human and the rat NK_1 receptor (Gerard et al., 1991; Fong et al., 1992a, b). Some evidence suggests that there are differences in the NK_1 receptor, even within a single species, as for example in different rabbit smooth muscle preparations (Beresford et al., 1991). It is also possible that alternative splicing produces two isoforms of the NK_1 receptor (Fong et al., 1992b). The expression of NK_1 receptor mRNA can be inhibited by glucocorticoids (Ihara and Nakanishi, 1990).

b. NK_2 RECEPTORS. The NK_2 receptor, at which the natural agonists have the rank order of potency NKA > NKB ≫ SP, also belongs to the family of G-protein-coupled rhodopsin-like receptors. The receptor protein contains 384 to 398 amino acid residues, depending on the species studied (Masu et al., 1987; Gerard et al., 1990; Sundelin et al., 1992). The N-terminally extended forms of NKA (NPK and NPγ) have higher apparent selectivity for the NK_2 receptor than does NKA. Thus, NPγ is approximately ten-fold more potent than NKA in contracting guinea pig and human bronchus (Black et al., 1990; Burcher et al., 1991). This is not seen in other tissues, however (Van Giersbergen et al., 1992). Studies using various ligands of both peptide and nonpeptide nature, including the selective NK_2 antagonist SR48968 (Emonds-Alt et al., 1992), indicate a heterogeneity among NK_2 receptors, especially between different species. The NK_2A type seems to be present in guinea pig and some human tissues (such as bronchi), whereas rat colon and vas deferens and hamster tissues contain NK_2B (Maggi et al., 1990a; Burcher et al., 1991; Advenier et al., 1992a, b; Delay-Goyet and Lundberg, 1991b; Lou et al., 1992b). However, these differences may be caused by species variations in receptor structure. Isolation of mRNA for different forms of the NK_2 receptor from the same species could settle finally the issue of NK_2 receptor heterogenity, but lack of such data does not rule out a possible subdivision, as posttranslational modifications may occur. Taken together, it seems clear that NK_2 receptors are heterogenous in their ability to recognize antagonists, but currently available data from studies using molecular biology do not support true intraspecies subdivision of NK_2 receptors.

c. NK_3 RECEPTORS. A 452-amino-acid residue receptor protein with higher affinity for NKB than for NKA and SP has been described and corresponds to the NK_3 receptor (Shigemoto et al., 1990; Buell et al., 1992). The rat isolated portal vein preparation is a classical monoreceptorial bioassay for NK_3 receptors in the periphery (Mastrangelo et al., 1987). Some peptide agonists, such as Senktide, are potent NK_3 stimulating agents, and a peptidic TK antagonist acts at NK_3 receptors (Stables et al., 1993). A selective nonpeptide receptor antagonist (SR142801) has recently been made available (Emonds-Alt et al., 1995; Patacchini et al., 1995). Because NKB does not seem to be present in primary sensory neurons, it is less likely that the NK_3 receptor is of general importance for the efferent function of sensory neurons, even if it should be emphasized that other TKs could also activate this receptor (table 2; fig. 1).

4. Tachykinin receptor antagonists: tachykinergic transmission. It is clear that in a number of peripheral tissues, sensory nerves release SP, NKA, and perhaps extended or truncated forms of NKA with NK_2 receptor preference. Furthermore, multiple TK receptors are present on target cells. Even if natural TKs have high affinity for certain receptors, there may be some degree of crosstalk (i.e., SP acting on NK_2 receptors and NKA acting on NK_1 receptors). As discussed below, potent and selective NK receptor antagonists have been used to define the relative roles of different TKs in sensory cotransmission (Lou et al., 1993; Satoh et al., 1993a, b), and TKs represent the first well defined peptide transmitters in the periphery. In the rat urinary bladder, the early phase contractile response to capsaicin may involve both NK_1 and NK_2 receptors, whereas the delayed tonic component is NK_2-mediated (Maggi et al., 1991a). In autonomic sympathetic ganglia, sensory axon collaterals mediate slow noncholinergic depolarization (EPSP) via TK release, presumably acting mainly on NK_1 receptors (Otsuka and Yoshioka, 1993; Zhao et al., 1993) (fig. 1). In airway parasympathetic ganglia, capsaicin evokes TK release from sensory nerves apparently acting on NK_3 receptors that facilitate ACh release (Myers and Undem, 1993), although this finding should be extended using the NK_3 receptor antagonist (SR142801). It should be emphasized that data obtained in vitro may not be directly comparable with the situation in vivo, in which peptide degradation is more likely to occur, especially degradation of SP (Martling et al., 1987). Furthermore, the use of NEP inhibitors may also change the natural patterns. Thus, both in guinea pig bronchus and isolated renal pelvis, the relative contribution by NK_1 receptors to the sensory nerve response was unmasked by peptidase inhibition, resulting in reduction of NK_2 antagonist efficacy (Maggi et al., 1991b, 1992). Finally, if NEP activity can change upon inflammation, the results in control animals may not necessarily translate to various animal disease models or to the situation in chronic human inflammation, e.g., in asthma, when airway epithelium containing NEP often is lost (Nadel, 1991).

When multiple D-amino acids are inserted in the backbone of SP, the resulting peptides often act as SP antagonists (Leander et al., 1981; Rosell and Folkers, 1982; Folkers et al., 1984). When these peptidic SP antagonists became available, they were used to establish the transmitter role of TKs for sensory nerve responses in peripheral tissues. TK-mediated responses include plasma protein extravasation (Lundberg et al., 1983a, c), bronchoconstriction (Lundberg et al., 1983a) and contraction of the iris sphincter (Leander et al., 1981; Björkroth, 1983). It became clear, however, that these SP antagonists also inhibited the effects of NKA (Karlsson

and Persson, 1985; Bailey et al., 1986). In addition to the poor selectivity, these early TK antagonists also had a number of other drawbacks: they had residual partial agonist activity (Bailey and Jordan, 1985), caused mast cell degranulation (Lundberg et al., 1983a), acted as local anesthetics (Post et al., 1985) and had neurotoxic effects (Freedman et al., 1988).

The second generation of TK receptor antagonists was also of peptidic nature but had improved potency and some selectivity for NK_1 or NK_2 receptors. Thus, L65977 had some selectivity for the NKA receptor (McKnight et al., 1988). GR82334 was a selective NK_1 receptor antagonist (Hagan et al., 1991), and MEN10207 analogs could separate NK_2A and NK_2B subtype responses (Rovero et al., 1990). The peptidic TK antagonists could be very small, even tripeptides (FR113680, Hagiwara et al., 1991) or dipeptides (FK888, Fuji et al., 1992). D-Actinomycin, an antitumor agent consisting of two cyclic peptides, can act as an NK_2 antagonist, presumably of NK_2A type, because it blocks the functional response to NKA, vagal stimulation or capsaicin in guinea pig bronchi but not in rat vas deferens (Delay-Goyet and Lundberg, 1991b; Fuji et al., 1991; Lou et al., 1992b). The NK_2 receptor-blocking activity of D-actinomycin analogs did not seem to be related to antitumor activity, however (Auberson et al., 1993).

The third generation of TK antagonists is of nonpeptide nature. The first of these, CP96345, displays a very high potency (nM) and selectivity in blocking NK_1 receptors (McLean et al., 1991; Snider et al., 1991). One obvious drawback with this drug is that in high concentrations, it depresses the contractile response to nonTK stimulants and lowers blood pressure in vivo (Delay-Goyet et al., 1992a), presumably caused by Ca^{2+} channel (Guard and Watling, 1993) blocking properties. Furthermore, in high concentrations, CP96345 depresses nerve-evoked contractions in vitro that are not TK-mediated and could be caused by interference with Na^+ channels (Wang and Håkanson, 1992; Caeser et al., 1993). The 2R,3R enantiomer CP96344, which is inactive as an NK_1 receptor antagonist, can be used to reveal this nonspecific effect of CP96345 (Delay-Goyet et al., 1992a; Lembeck et al., 1992). CP96345 was found to be effective in blocking the plasma protein extravasation evoked by cigarette smoke in rat airways (Delay-Goyet and Lundberg, 1991a) or the extravasation response to capsaicin (Eglezos et al., 1992), antidromic nerve stimulation and mustard oil (Lembeck et al., 1992). Because the inactive enantiomer CP96344 did not influence, for example, cigarette smoke-evoked plasma protein extravasation in the trachea, it seems clear that NK_1 receptors are involved in the inflammatory responses mediated by endogenous TKs released from primary afferent nerves (Delay-Goyet et al., 1992a). Furthermore, the adhesion of neutrophils and eosinophils in rat trachea induced by SP, capsaicin and hypertonic saline is inhibited by CP96345, also suggesting the importance of NK_1 receptor mechanisms (Baluk et al., 1995). The bronchoconstriction responses to capsaicin is, however, not or only marginally influenced by CP96345 (Ballati et al., 1992a; Lou et al., 1992b): therefore, even if NK_1 receptors are present on bronchial smooth muscle, they do not normally seem to be activated by endogenous TKs (Lou et al., 1993). As discussed above, RP67580 is much more active on rat than on human NK_1 receptors. This may be attributable to species differences in binding epitopes between the NK_1 receptors (Fong et al., 1992a, b, 1993). RP67580 is also a very potent inhibitor of neurogenic plasma protein extravasation in rat skin (Garret et al., 1991) and airway mucosa (Auberson and Lundberg, 1993; Delay-Goyet et al., 1993), while having no effect on vasodilation in the skin in response to antidromic nerve stimulation (fig. 3) (Delay-Goyet et al., 1992b, 1993; Kerezoudis et al., 1994). It has been reported, however, that capsaicin-evoked vasodilation in rat nasal mucosa is reduced by the NK_1 antagonist CP99994, suggesting tissue differences (Piedimonte et al., 1993).

The first NK_2 receptor antagonist SR48968 (Emonds-Alt et al., 1992) potently antagonizes TK effects on NK_2 receptors, in guinea pig and humans (Advenier et al., 1992a, b; Lou et al., 1993). SR48968 markedly inhibits capsaicin- and nerve stimulation-evoked sensory bron-

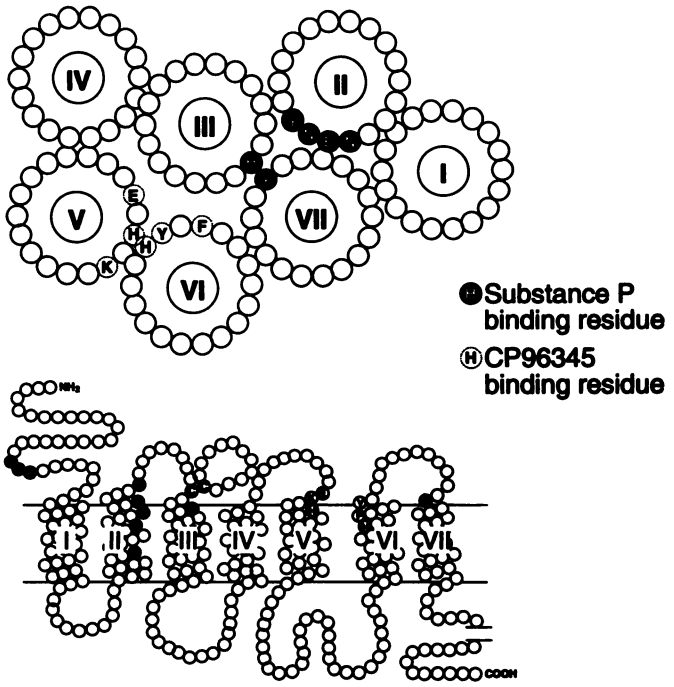

FIG. 5. A helical wheel (*upper panel*) and a serpentine (*lower panel*) diagram of the human NK_1 receptor. The helical wheel model shows the outer portions of the helices and presumed ligand contact sites with different amino acid residues of the receptor. The serpentine diagram shows seven transmembrane regions with extracellular and intracellular domains. Based on point mutations, amino acid residues suggested to be involved in the binding of the natural peptide agonist, SP, are shown in white on black, and residues involved in the binding of the nonpeptide antagonist CP96345 are shown in black on white (modified from Rosenkilde et al., 1994).

choconstriction in the guinea pig, further illustrating the importance of NK_2 receptors for this response (fig. 4) (Lou et al., 1993; Satoh et al., 1993a). Furthermore, SR48968 antagonizes citric acid aerosol-evoked bronchoconstriction in this species (Satoh et al., 1993b). Interestingly, SR48968 also blocks the bronchoconstrictor effects of SP on human bronchi (Advenier et al., 1992a); a large portion of the NKA-evoked bronchoconstriction is still present in the guinea pig, in contrast to the response to selective NK_2 agonists such as NKA(4–10), after this agent (Lou et al., 1993), illustrating the problem with only relative NK receptor selectivity of endogenous TKs. The "innervated" smooth muscle NK receptor in guinea pig bronchi is of the NK_2 type, as revealed by the marked inhibitory effects of the nerve stimulation response by SR48968. Recent data have also suggested that NKA-evoked plasma protein extravasation in distal guinea pig airways is blocked by SR48968 (Tousignant et al., 1993).

A number of nonpeptide antagonists have appeared after CP96345. Two subsequent contributions to the arsenal of antagonists are SR140333 (Emonds-Alt et al., 1993) and RPR100893 (Tabart and Peyronel, 1994; Fardin et al., 1994) that represent highly selective and potent NK_1 antagonists. It is of interest that, also after treatment with SR140333, the sensory vasodilation response to capsaicin in the pig skin and nasal mucosa is largely unaffected, whereas the vasodilator response to exogenous SP is abolished (Rinder and Lundberg, 1996).

5. Competitive nonpeptide antagonists act allosterically. Interesting information has emerged concerning amino-acid-binding epitopes for nonpeptide NK receptor antagonists, which differ from the binding epitopes for the endogenous TK ligands (fig. 5). Thus, recent data using site-directed mutagenesis suggest differences between binding and functional sites in the NK receptors. Nonconserved residues in two epitopes close to the top of transmembrane segment V—and, in one epitope, at the top of segment VI of the NK_1 receptor—are essential for binding of the nonpeptide antagonist CP96345 but are not important for the interaction with SP or the peptide antagonist Spantide with the NK_1 receptor (Gether et al., 1993a). Histidine residue 197 is probably involved in an amino-aromatic interaction with CP96345, whereas this amino acid has no role for the SP binding (Fong et al., 1993). Furthermore, another nonpeptide antagonist, RP67580, is not affected by mutation of histidine 197 (Fong et al., 1992a, b). Therefore, nonpeptide and peptide antagonists seem to interact with NK_1 receptors in different ways. The large peptide analogs compete with natural ligands (SP) for sites located in the extracellular domain of the receptor (Fong et al., 1992c), whereas small nonpeptidic components enter the small hydrophobic domain created by transmembrane segments V to VII and interfere with the activation of the receptor by SP (Gether et al., 1993b). The agonist (SP) may still bind, but the NK_1 receptor is not activated (Gether et al., 1993a, b, c) because of "negative intrinsic activity" (see Schütz and Freissmuth, 1992) that may result from receptor inactivation either following G-protein uncoupling or because of an alteration of the general structure of the receptor that shifts it away from the agonist binding conformation. Substitutions in transmembrane segment II in the NK_1 receptor do not affect the high affinity binding of SP but instead attenuate the ability of TK peptides to compete for nonpeptide antagonist binding. Thus, certain mutations in the receptor may impair interchange between receptor conformations such that each bind to different ligands with high affinity, whereby agonists stabilize active and antagonists stabilize inactive receptor conformations (fig. 5) (Rosenkilde et al., 1994). Because large peptide agonists may not reach into the binding pocket of nonpeptide antagonists, a situation exists in which two ligands compete for the same receptor but use different binding epitopes. The nonpeptide antagonists can therefore act as allosteric competitive antagonists. Finally, it should be emphasized that receptor mutagenesis studies are associated with problems of interpretation, because changes in binding may not always represent a true hit, i.e., revealing contact residues between charged amino acids in the receptor and the ligand. Thus, indirect hits may occur because of structurally important residues; more remote hits and pseudohits may be explained by influence on conformational interchange between receptor states. Furthermore, point mutations can evoke reduced expressions of the receptor protein.

6. Tachykinins and sensory pathophysiology. In the periphery, NK_1 receptor antagonists may be useful for the initial treatment of edema caused by thermal injury (Saria and Lundberg, 1983; Saria, 1984). TKs have been suggested to be involved in the pain and inflammation evoked by rheumatoid arthritis, and the SP content is increased in sensory neurons in adjuvant-induced polyarthritis (Colpaert et al., 1983; Schoenen et al., 1985). Furthermore, SP induces collagenase release from rheumatoid synoviocytes and stimulates their proliferation (Lotz et al., 1987). Animal models have implied that TKs could have a role in the pathogenesis of airway disease (Saria et al., 1988). Airway infections cause long-lasting potentiation of neurogenic inflammation (plasma protein extravasation) in the lower airways (Piedimonte et al., 1990; McDonald et al., 1991), which is a NK_1 receptor-mediated response. Clearly, TKs can cause bronchoconstriction, airway edema and mucus secretion in addition to inflammatory cell changes (Braunstein et al., 1991), which implies that NK receptor antagonists may be relevant in treatment of airway diseases. Furthermore, patients with airway disease are often hyperreactive to inhaled irritants, such as cigarette smoke, which are known to activate capsaicin-sensitive nerves and evoke release of TKs (Lundberg and Saria, 1983; Lee et al., 1995). SP may also be released upon allergen provocation in the nose (Nieber et al., 1991, 1992) and lower

airways (Germonpré et al., 1995), and it is clear that allergen challenge in human skin activates capsaicin-sensitive nerves (Lundblad et al., 1987a). In humans, TKs have been shown to increase plasma exudation in nasal mucosa, but whether neurogenic plasma protein extravasation also occurs in lower airways still remains to be proven (Germonpré et al., 1995). The late plasma protein extravasation response in guinea pig trachea upon allergen challenge was inhibited by the NK_1 antagonist CP96345 (Bertrand et al., 1993), in agreement with earlier data using capsaicin pretreatment (Saria et al., 1983b). Inhibition of SP degradation reverses steroid-induced suppression of neutrophil adhesion on rat tracheal blood vessels, suggesting involvement of neurogenic mechanisms (Katayama et al., 1993). The expression of NK_1 receptors is increased in asthmatic lungs and reduced after treatment by glucocorticosteroids (Adcock et al., 1993). Furthermore, there may be a proliferation of sensory SP-IR nerves in patients with lethal asthma (Ollerenshaw et al., 1991), although this is not the case in milder forms of the disease (Howarth et al., 1991). Whereas NK_1 receptors primarily mediate proinflammatory responses in the airways, NK_2 receptor activation increases bronchial smooth muscle tone (Advenier et al., 1992a; Satoh et al., 1992). Therefore, agents with combined NK_1 and NK_2 blocking activity, such as FK224, may be more efficacious than highly selective compounds in treatment of airway disease. FK 224 failed to block the bronchoconstrictor effect of NKA in patients with asthma, however, and more potent nonpeptide antagonists, such as SR48968, should also be tested in humans (Howarth et al., 1991; see Joos et al., 1995). It may also be advantageous to antagonize the long-term effects of TKs, such as cell proliferation, although it will be important to establish whether chronic treatment with NK receptor antagonists will lead to changes in tissue integrity and repair concerning the trophic actions of TK peptides. After capsaicin desensitization, antigen-induced bronchospasm is attenuated in sensitized guinea pigs (Manzini et al., 1987). Inhibition of NEP potentiates bronchoconstriction by antigen in guinea pigs (Kohrogi et al., 1991). Antigen challenge not only causes acute TK release from the lung (Saria et al., 1988) but also evokes a long-lasting potentiation of TK secretion (Ellis and Undem, 1993). Presumably, both peptidoleukotrienes (Ellis and Undem, 1991; Martins et al., 1991) and histamine (Saria et al., 1988; Ellis and Undem, 1993) may be involved in this facilitation of sensory neuropeptide release. Furthermore, capsaicin pretreatment prevents the induction of airway hyperresponsiveness in a guinea pig model of asthma (Ladenius and Biggs, 1989; Matsuse et al., 1991). Repeated antigen challenges induce airway hyperresponsiveness to NKA and vagal sensory bronchoconstriction in the guinea pig (Ballati et al., 1992b). In accord, inhaled NKA is more efficient as a bronchoconstrictor in airways of patients with asthma than in normal subjects (Joos et al., 1987).

Human airways are very sensitive to the irritant effects of capsaicin (Stjärne et al., 1989a), and patients with vasomotor rhinitis have an increased reflexogenic secretory response to capsaicin (Stjärne at al., 1989a; Lacroix et al., 1991), an elevated nasal mucosal content of sensory neuropeptide (Lacroix et al., 1992) and reaction to SP (Devillier et al., 1988). In addition, the cough sensitivity to capsaicin in humans increases during viral infections (O'Connell et al., 1993). This observation is of interest in view of the the data suggesting that the cough reflex in guinea pigs to citric acid is inhibited by the NK_2 antagonist SR48968 (Advenier et al., 1993). Although capsaicin is a potent tussive agent in humans, the bronchoconstriction response to inhaled capsaicin is much more transient than in the guinea pig and is atropine-sensitive (Fuller et al., 1985). It is of interest that patients treated with ACE inhibitors develop cough, and the cough response to capsaicin is enhanced in these patients (Fuller and Choudry, 1987). Local desensitization with capsaicin leads to marked and prolonged reduction of symptoms of congestion and secretion in patients with vasomotor rhinitis (Lacroix et al., 1991, 1992; Stjärne et al., 1991b). The possible involvement of TKs for the beneficial effects of capsaicin desensitization in this condition remains to be established.

TKs have also been implicated in inflammatory diseases of the eye, especially in the rabbit, where they cause breakdown of the blood-aqueous barrier, increased intraocular pressure and iris sphincter contraction. However, the variations between species are large, and the situation in humans is still unclear (Andersson, 1990). Prostaglandins may excite sensory C-fibers upon eye trauma because indomethacin reduces SP levels in ocular aqueous humor upon surgery (Kieselbach et al., 1993).

TKs may also be involved in bladder disease. Thus, NK_2 receptor antagonists have beneficial effects in a model of chemically (xylene) induced cystitis in the rat by a mechanism that involves sensory nerves (Pietra et al., 1992). TKs may possibly also influence bladder reflexes, probably at the spinal cord level (Lecci et al., 1992, 1993). Capsaicin-sensitive afferents are also present in human bladder, and their stimulation produces pain and hyperreflexia (Maggi et al., 1989a), suggesting that TK antagonists may be of use in the treatment of cystitis.

It has been suggested that TKs are involved in conveying sensory information to the CNS (fig. 1). Nonpeptide NK_1 receptor antagonists reduce the pain response, especially to chemical stimuli (Garret et al., 1991; Yamamoto and Yaksh, 1991); NK_2 receptors may also be involved (Urban et al., 1992). Furthermore, TK antagonists may be of value as antimigraine agents, providing that plasma protein extravasation is an important component of the pathophysiology in humans (Buzzi et al., 1991). Another obvious clinical target for nonpeptide TK receptor antagonists is patients with carcinoid tumors,

which often secrete large amounts of SP, NKA and NPK into the systemic circulation (see Theodorsson et al., 1985, 1987). Two cardinal symptoms, i.e., skin flush and bronchoconstriction, may be related to circulating tachykinins acting via NK_1 and NK_2 receptors, respectively.

D. Calcitonin Gene-related Peptide as Neurotransmitter

1. Synthesis, release and degradation. Calcitonin gene-related peptide (CGRP) is present in sensory neurons (fig. 1) (Rosenfeld et al., 1983), and subsequent characterization has revealed at least two forms, CGRPα and β, with some species variations in amino acid composition (Morris et al., 1984; Amara et al., 1985). CGRPα seems to be the predominant form in sensory neurons (Gibson et al., 1988). CGRP is transported axonally to terminal areas in, for example, visceral organs (Green and Dockray, 1988) (table 2). TKs and CGRP are present together in capsaicin-sensitive sensory nerves in the skin and internal organs (fig. 1) (Gibbins et al., 1985; Lundberg et al., 1985a; Martling et al., 1988). Systemic capsaicin pretreatment causes loss of both TKs and CGRP in nerves in airways and other tissues (Gibbins et al., 1985; Lundberg et al., 1985a; Forsberg et al., 1988). There is also ultrastructural evidence that TKs and CGRP are costored in the same large dense cored vesicles in sensory neurons (Gulbenkian et al., 1986; Merighi et al., 1988), and these peptides are coreleased upon activation of sensory nerves by, for example, capsaicin (Saria et al., 1986; Martling et al., 1988). In the guinea pig, the overlap between TK and CGRP in sensory neurons is virtually complete, but it should be emphasized that in other species, CGRP neurons may in some cases lack TKs (Holzer, 1988; Hökfelt et al., 1994). CGRP is more metabolically stable than TKs (Katayama et al. 1991) and therefore easier to detect; thus, it is useful as marker of peptide release from capsaicin-sensitive nerves (Lou, 1993). NEP inhibition by phosphoramidon reduces CGRP degradation (Kröll et al., 1990; Katayama et al. 1991) and somewhat enhances CGRP outflow (although to a much smaller extent than the well known effect on TKs) (fig. 4) and it enhances certain CGRP-evoked functional responses as well (Maggi and Giuliani, 1994).

2. Biological actions. a. VASCULAR EFFECTS CGRP is a potent vasodilator in a variety of vascular beds of different species (Brain et al., 1985; Lundberg et al., 1985a), including humans (Franco-Cereceda et al., 1987b, c). Typically, the effect is very long-lasting, for example, when the peptide is injected into the human skin (Pietrowski and Foreman, 1986). In contrast to TKs, CGRP exerts direct vasodilatory actions on vascular smooth muscle, and the effect is usually endothelium-independent (Franco-Cereceda et al., 1987a) except in certain large vessels, such as the aorta (Gray and Marshall, 1992). CGRP has been reported to activate ATP-sensitive K^+ channels in arterial smooth muscle (Nelson et al., 1990), leading to hyperpolarization. These channels are also activated by the K^+ channel opener, chromakalin, and blocked by the sulphonylurea type antidiabetic agent, glibenclamide, reducing the hypotension in vivo (Andersson, 1992) or relaxation of lingual artery in vitro to CGRP (Kobayashi et al., 1995). In contrast, activation of ATP-sensitive K^+ channels does not appear to mediate relaxation in other arteries in response to CGRP (Prieto et al., 1991; Zschauer et al., 1992). In the nasal mucosa, there is evidence that CGRP dilates both arteriolar resistance vessels and capacitance vessels regulating blood flow and mucosal volume, respectively (Stjärne et al., 1989b, 1991a).

CGRP does not per se evoke plasma protein extravasation but can enhance the response to TKs (Gamse and Saria, 1985). CGRP can degrade the blood-aqueous barrier, causing leakage of plasma proteins in the eye, however (Wahlestedt et al., 1986), and also this response seems to be inhibited by glibenclamide (Andersson, 1992).

b. INCREASED CARDIAC CONTRACTILITY. CGRP causes increased contractility (positive chronotropic and inotropic effect) of the right atrium of a variety of species, including humans (Franco-Cereceda and Lundberg, 1985; Saito et al., 1986; Franco-Cereceda et al., 1987c). In electrophysiological studies, CGRP was shown to prolong the myocyte action potential in guinea pig atria, much in the same way as β-receptor stimulation (Franco-Cereceda et al., 1988). The CGRP effect is independent of β-adrenoceptors, however, and is observed also in vivo (Franco-Cereceda et al., 1987b, c, 1988; see Franco-Cereceda, 1988).

c. RELAXATION OF NONVASCULAR SMOOTH MUSCLE. CGRP causes relaxation of smooth muscle in a variety of tissues such as the ureter (Hua and Lundberg, 1986) and vas deferens (Maggi et al., 1987b), whereas no major influence is exerted upon bronchial smooth muscle (Martling et al., 1988). In the ureter, CGRP seems to activate ATP-sensitive K^+ channels (Maggi et al., 1994a).

d. EFFECTS ON CARBOHYDRATE METABOLISM. CGRP is a potent inhibitor of insulin-mediated glycogen synthesis in skeletal muscle with effects in the low nM range, whereas other sensory neuropeptides, such as SP, are inactive in this aspect (Leighton and Foot, 1995). Whether this effect also is influenced by the blood glucose-lowering agent glibenclamide remains to be established.

e. IMMUNOLOGICAL EFFECTS AND TROPHIC ACTIONS. CGRP can cause accumulation of leucocytes in the human skin (Pietrowski and Foreman, 1986) and can increase cell proliferation, such as of human endothelial cells (Hägerstrand et al., 1990). Furthermore, CGRP may enhance neutrophil accumulation and the inflammatory actions of other mediators, such as interleukin 1 (Buckley et al., 1991). In another peripheral neuronal system, the cholinergic α-motor neurons, CGRP may represent an anterograde factor, which, after release at

the motor end-plate, regulates gene-expression of the nicotinic N_1-receptors (Fontaine et al., 1987).

3. Calcitonin gene-related peptide receptors and antagonists. Although the CGRP receptor proteins have not yet been cloned, indirect evidence suggests that they belong to the superfamily of G-protein-coupled receptors and increasing evidence suggest a heterogeneity of the CGRP receptors: (*a*) there are differences in the relative efficacy and potency of CGRP analogs (Dennis et al., 1990; Quirion et al., 1992), (*b*) CGRP stimulates cAMP formation (Sigrist et al., 1986), but the receptors vary in their coupling to adenylate cyclase (Stangl et al., 1993), (*c*) their molecular weight ranges from 54.000 to 90.000, partly due to tissue-specific glycosylation (Stangl et al., 1991, 1993). CGRP can stimulate adenylyl cyclase and increase the intracellular content of cAMP in rat aortic smooth muscle cells (Kubota et al., 1985). There is also support for the possibility that a cAMP-dependent mechanism may be involved in activation of K_{ATP} channels in myocytes (Notsu et al., 1992). Based on pharmacological criteria, and using the antagonist CGRP fragment CGRP(8–37), CGRP receptors have been characterized into two subtypes (Chiba et al., 1989; Qurion et al., 1992) (table 2). $CGRP_1$ receptors are readily blocked by CGRP (8–37) whereas $CGRP_2$ receptors are far less sensitive. Neither human α-, nor human β-CGRP(8–37) shows selectivity for supposedly $CGRP_1$ (guinea pig atrium) or $CGRP_2$ (rat vas deferens) (Dennis et al., 1989, 1990) receptor-mediated responses, however (Longmore et al., 1994).

4. Calcitonin gene-related peptide-ergic transmission. Increasing evidence suggests that sensory nerve-evoked vasodilation is mediated via CGRP. Thus, CGRP mimics capsaicin-evoked vasodilation of isolated blood vessels in vitro; both responses are independent of the endothelium in contrast to the effects of TKs (Franco-Cereceda et al., 1987a). CGRP is likely to mediate the sensory endothelium-independent NANC relaxant response in pulmonary arteries of the guinea pig (Maggi et al., 1990b; Liu et al., 1992a). Furthermore, CGRP (8–37) inhibits the capsaicin-evoked relaxation of porcine coronary arteries without influencing the effect of TKs (Franco-Cereceda, 1991). Also, in the isolated canine lingual artery, CGRP released from sensory nerves by EFS produces endothelium-independent relaxation via activation of $CGRP_1$ receptors. Interestingly, glibenclamide inhibited both CGRP- and EFS-evoked relaxations, suggesting involvement of K_{ATP} channels (Kobayashi et al., 1995). Also, it has been found in in vivo studies that CGRP (8–37), when given systemically, reduces local blood flow in many vascular beds and induces hypertension (Gardiner et al., 1990a). This suggests that endogenous CGRP release may evoke a basal vasodilator tone. In rat oral tissues (Kerezoudis et al., 1994) and skin (Delay-Goyet et al., 1992b), CGRP (8–37) had no effect on basal blood flow, however. In rabbit (Hughes and Brain, 1991), rat (Delay-Goyet et al., 1992b), pig skin and nasal mucosa (Rinder and Lundberg, 1996) and rat oral tissues (Kerezoudis et al., 1994), the vasodilation evoked by electrical antidromic nerve stimulation or capsaicin are markedly reduced by CGRP (8–37), suggesting involvement of $CGRP_1$ receptor responses (fig. 3). CGRP (8–37) markedly reduced the duration of the sensory vasodilation response, while a rapid initial increase in blood flow still remained. This suggests that also other mechanisms (such as $CGRP_2$ receptors or other mediators) are involved in antidromic vasodilation. TKs would also be possible mediator candidates of rapid vasodilation effects, but NK_1 or NK_2 antagonists do not reduce antidromic (fig. 3) or capsaicin-evoked vasodilation, thus excluding TKs as major additional mediators (Delay-Goyet et al., 1992b; Escott and Brain, 1993; Rinder and Lundberg, 1996). NOS inhibition, if anything, enhanced the trigeminal vasodilation in oral tissues (Kerezoudis et al., 1993a). Unlike the parasympathetic neurons, these sensory nerves probably do not contain high levels of NOS activity, however (Kerezoudis et al., 1993b). In contrast, NOS inhibitors attenuate antidromic or capsaicin-evoked vasodilation in the gastric mucosa (Whittle et al., 1992; Holzer et al., 1993b). This may possibly be related to the fact that visceral spinal afferents contain NOS (or rather NADPH diaphorase) (Aimi et al., 1991). Based on experiments in the rat skin, Holzer and Jocic (1994) concluded that NO does not play a vasorelaxant messenger role in antidromic nerve stimulation-evoked vasodilation but can contribute to activation of, and/or transmitter release from sensory fibers in response to chemical irritation by mustard oil. Furthermore, sodium nitroprusside, which releases CGRP from sensory nerves (Wei et al., 1992), caused hyperemia that was significantly diminished by the CGRP antagonist CGRP(8–37) (Holzer and Jocic, 1994). Also, in the rabbit skin, NO seems to release CGRP; interestingly, even capsaicin may stimulate NOS (Hughes and Brain, 1994). In fact, capsaicin-induced and bradykinin-induced production of cGMP in cultured primary afferent neurons is blocked by an inhibitior of NOS (Bauer et al., 1993). This suggests that NO can be formed at least in some stimulated afferent neurons and may play an important role in the chemical activation of sensory neurons and subsequent release of peptide mediators (fig. 2). Interestingly, increasing evidence suggests that the neurogenic hyperemia in rat skin induced by locally administered NO donors involves formation of prostaglandins that in turn cause release of CGRP from afferent nerve fibers (Holzer et al., 1995). The NO donor sodium nitroprusside can facilitate the stimulus-evoked release of CGRP from sensory neurons in culture, although it is unable to release on its own (Dymshitz and Vasko, 1994). This may suggest that NO does not directly act on sensory neurons but requires intermediate messengers such as COX products that are formed in sufficient quantities in the skin but not in cultures of afferent nerves. NO has in fact recently been

shown to stimulate the formation of prostaglandins in the rat forepaw (Sautebin et al., 1995). Furthermore, the cutaneous vasodilation by PGE_2 depends on CGRP release without involving formation of NO or COX activation (Holzer et al., 1995) (fig. 2).

The role of CGRP as mediator of capsaicin-evoked positive inotropic and chronotropic effects on the atrium is also well substantiated. Thus, the capsaicin response is abolished after CGRP tachyphylaxis (Franco-Cereceda and Lundberg, 1985; Saito et al., 1986) or after treatment with CGRP (8–37), also in vivo (Satoh et al., 1993) (fig. 4).

Capsaicin-evoked inhibition of motility in the renal pelvis, ureter, uterus (Zernig et al., 1984), and vas deferens is also most likely caused by CGRP, as revealed by studies using CGRP (8–37) (fig. 4) (Maggi et al., 1992, 1994a; Satoh et al., 1993) or immunoneutralization with antisera to CGRP (Maggi et al., 1987c). In the guinea pig ureter, the ability of both exogenous and endogenous CGRP to suppress spontaneous contractions through electro-mechanical coupling seem to involve the activation of glibenclamide-sensitive (K_{ATP}) potassium channels (Maggi et al., 1994a; Santicioli and Maggi, 1994). The membrane hyperpolarization produced by activation of the K_{ATP} channels is capable of suppressing the evoked motility sustained by depolarization of latent pacemakers in the ureter (Maggi et al., 1994a). Possibly, locally released CGRP may prevent antiperistalsis in the ureter (fig. 4). Also, in the human ureter, CGRP inhibits contractile activity (Hua et al., 1987), although it should be emphasized that in humans, the density of CGRP nerves is much lower than in the guinea pig (Hua et al., 1987). Capsaicin-evoked CGRP release from sensory nerves in skeletal muscle has recently been postulated to counterregulate insulin effects in skeletal muscle (Leighton and Foot, 1995).

5. Calcitonin gene-related peptide and pathophysiology. As discussed above, there is ample evidence that CGRP is involved in the axon reflex vasodilation seen upon stimulation of sensory nerves. Because allergic reactions cause activation of capsaicin-sensitive nerves both in the skin (Lundblad et al., 1987a) and airways (Alving et al., 1990), development of a nonpeptide CGRP antagonist would be of interest to block vasodilatory responses upon anaphylaxis. Also, in migraine, which involves cerebral vasodilation associated with marked activation of pain fibers, a CGRP antagonist may be of value. It is of interest that sumatriptan, a 5HT-1_D agonist that prevents dilation of cerebral blood vessels and is a very efficient antimigraine therapeutic drug, inhibits CGRP release evoked by trigeminal nerve stimulation (Buzzi et al., 1991). Furthermore, elevated plasma levels of CGRP in the jugular vein of patients upon migraine (Goadsby and Edvinsson, 1993) and cluster headache attack evoked by nitroglycerine (Fanciullacci et al., 1995) are reduced by sumatriptan in parallel with relief of pain symptoms. Because nitroglycerine is a classical NO donor, the relation between COX activation and CGRP release should be investigated (Salvemini et al., 1993). In the guinea pig, bronchus preparation sumatriptan did not inhibit sensory neuropeptide release, however, as revealed by functional analysis of the NANC response (Ward et al., 1994). Another aspect on the role of CGRP is the finding that CGRP receptor antagonist peptide exacerbates hypoxic pulmonary hypertension in rats exposed to chronic hypoxia, suggesting that endogenous CGRP serves as a counterregulating factor in this condition (Tjen-A-Looi et al., 1992). It is also of interest that perivascular CGRP fibers decrease with age in spontaneously hypertensive rats (Kawasaki et al., 1990). Acid back-diffusion through a disrupted gastric mucosal barrier leads to a rise in blood flow in the stomach mucosa which limits acid damage to the mucosal surface (Holzer et al., 1991). This acid-evoked hyperemia in rat gastric mucosa is inhibited by CGRP(8–37) (Li et al., 1992) but not by the NK_1 receptor antagonist RP67580 (Holzer et al., 1994). During severe hypo-glycemia, release of CGRP from sensory nerves in skeletal muscle may accelerate the recovery of blood glucose levels to normal (Leighton and Foot, 1995). Whether diabetic patients that are treated with sulphonylureas have a deficient CGRP function remains to be studied.

There is evidence that CGRP synthesis is upregulated upon inflammation. Both in guinea pig lower airways where inflammation was induced by chronic cigarette smoke exposure (Karlsson et al., 1991) and in human patients with vasomotor rhinitis the mucosal content of CGRP is increased (Lacroix et al., 1992). During adjuvant-induced inflammation of the hind paw, synthesis and release of CGRP is enhanced as revealed by mRNA changes in spinal ganglia and peptide levels in spinal cord, respectively (Galeazza et al., 1995). After axonal injury sensory neurons reduce their CGRP production, just as for TKs, however (Hökfelt et al., 1994).

E. Tachykinin and Calcitonin Gene-Related Peptide Cotransmission

1. Prejunctional interactions. There is no clear functional or biochemical evidence suggesting that NK_1, NK_2 or $CGRP_1$ receptor antagonists modify peptide release from peripheral branches of sensory neurons (Lou et al., 1993, fig. 4). Furthermore, NK_1 antagonists generally do not modify the vasodilatory response to CGRP (see Rinder and Lundberg, 1996).

2. Postjunctional interactions. In a variety of instances CGRP and TKs exert synergistic effects. Thus, SP and CGRP cause supra-additive vasodilation when given together (Gazelius et al., 1987). Furthermore, CGRP enhances TK-evoked plasma protein extravasation (Gamse and Saria, 1985). In the heart, however, the effects of NKA are opposite to those of CGRP, although it is not clear whether endogenous NKA also can exert this action (Franco-Cereceda and Lundberg, 1988). In the ure-

ter (Hua and Lundberg, 1986) and kidney pelvis (Maggi et al., 1992) TKs and CGRP also exert opposite effects, inducing contraction and relaxation respectively (fig. 4). There is evidence from the guinea pig suggesting that a low degree of nerve activation mainly inhibits ureter motility via CGRP, while strong activation causes contraction via TKs (Hua and Lundberg, 1986). The same sensory nerve can thus release two mediators with opposing actions and this effect may be related to different susceptibility of these peptides to peptidases, whereby CGRP is considerably more stable than NKA or SP. NEP inhibition thus enhances the contractile component to capsaicin in the ureter which is TK mediated (fig. 4). Maggi and Giuliani (1994) recently reported that a thiorphan-sensitive mechanism regulates the action of both endogenous and exogenous CGRP in the ureter. In the bronchus, TKs and capsaicin cause contraction, while CGRP seems to be largely without effect on bronchial smooth muscle. It is possible that CGRP increases local blood flow, however, contributing to the energy supply during smooth muscle activation (Matran et al., 1989a). This conclusion about bronchial mechanisms is also supported by autoradiographic studies which show that TK and CGRP receptors differ in their distribution (Carstairs and Barnes, 1986; Carstairs, 1987). In the rat vas deferens, the main effect of capsaicin is relaxation (presumably via CGRP) (Maggi et al., 1987b). Also NK_2 receptors are present in this organ and stimulate contraction but these receptors may not be activated by endogenously released TKs. In the mouse vas deferens, sympathetic control may be inversely modulated by TKs and CGRP released from sensory nerves acting via NK_1 and $CGRP_1$ receptors, respectively (Parlani et al., 1995). Both TKs and CGRP may also be involved in gastric mucosal protection against ulceration (Holzer, 1988).

Regarding more long-term aspects on TK and CGRP mechanisms, as mentioned above chronic activation of sensory mechanisms upon inflammation leads to enhancement of both TK and CGRP synthesis. In contrast, after peripheral axonal injury (transection), primary sensory neurons suppress production of excitatory transmitters including TKs and CGRP and switch to production of molecules likely to be of importance for survival and recovery which have been transiently expressed during development, such as NPY and VIP (Suburo et al., 1992; Hökfelt et al., 1994).

F. Glutamate and Peptidergic Afferent Transmission to Central Nervous System

Since afferent neurons contain glutamate, a likely main transmitter of sensory information to the CNS (table 2) (De Biasi and Rustioni, 1988; Merighi et al., 1991) there may be a variety of interactions between neuropeptides and glutamate, both at the peripheral branches and at the central synapse of capsaicin-sensitive neurons (fig. 1). There is some evidence for the presence of peripheral excitatory glutamate receptors of the kainate type (Ault and Hildebrand, 1993). Thus, in primary afferent neurons, kainate (KA) receptors are probably restricted to C-fiber axons and cell bodies and capsaicin depolarizes KA-sensitive fibers. In contrast to the effect of capsaicin, the KA-evoked depolarization is not blocked by ruthenium red (Ault and Hildebrand, 1993). Peripheral KA receptors on C-fibers may be activated either by glutamate released from damaged cells or macrophages (Piani et al., 1991) or by glutamate released upon depolarization of sensory neurons by other stimuli, subsequently activating prejunctional facilitatory autoreceptors (fig. 1). The depolarizing action of capsaicin is not modified by a KA receptor antagonist, however (Ault and Hildebrand, 1993) and the influence of KA receptor activation on peripheral neuropeptide release remains to be studied. It seems clear, however, based on experiments with peptide receptor antagonists, that glutamate is not the transmitter of the predominantly long-lasting effects described above, related to axon reflexes and "motor" functions of sensory nerves in the peripheral nervous system (fig. 1). However, other possible roles for glutamate released as a rapidly acting transmitter from peripheral capsaicin-sensitive sensory nerves remain to be examined.

Regarding transmission from the central branch of primary afferent neurons to the CNS, it is likely that an L-glutamate-mediated fast EPSP is accompanied by a TK-mediated slow EPSP in second order neurons in the dorsal horn of the spinal cord (Jessell et al., 1986). Capsaicin activation of spinal sensory neurons leads to release of tachykinins, CGRP (Saria et al., 1986) and glutamate (Ueda et al., 1995a, b). Both NMDA and alpha-amino-methyl-isoxazole-proprionic acid (AMPA) receptors are involved in sensory nociceptive transmission in mammalian spinal cord, but during infrequent synaptic activity glutamate activates mainly AMPA receptors (Wilcox, 1991) (fig. 1). Experimental evidence suggests that NMDA and nonNMDA receptor mechanisms may act preferentially in spinal transmission of cutaneous and muscular nociception, respectively (Song and Zhao, 1993). Somatosensory stimuli release tachykinins from primary afferent neurons that terminate in the spinal cord. This activates neuronal NK_1 receptors which, like the NK_1 receptors on postcapillary venules (Bowden et al., 1994), are internalized by endocytosis and lead to structural reorganization in the CNS (Mantyh et al., 1995). This NK_1 receptor internalization and dendritic structural reorganization in the dorsal horn of the spinal cord produce a specific image of neurons activated by sensory tachykinins and suggest mechanisms of desensitization and neuronal plasticity (Mantyh et al., 1995).

Involvement of both SP (NK_1) and NMDA receptor mechanisms has been demonstrated in spinal facilitation of sensory transmission i.e., "wind up" produced by repetitive stimulation of C-fiber afferents (Kellstein et al., 1990; Xu et al., 1991; Laird et al., 1993). Thus,

intrathecal application of an NK_1 antagonist or an NMDA receptor antagonist reduces facilitation of the flexor reflex (Xu et al., 1991). NK_2 receptors may also be involved in the central sensitization process (Nagy et al., 1993). Taken together, there is considerable evidence that also SP is involved in central pain transmission, although probably not as a primary rapidly acting transmitter (Otsuka and Yoshioka, 1993). There is conflicting evidence in the literature regarding the effects of various nonpeptide NK_1 and NK_2 receptor antagonists on central pain transmission, at least partly due to nonspecific effects such as Ca^{2+} blockade by these agents when used in very high doses (see Couteix et al., 1993; Rupniak et al., 1993; Yashpal et al., 1993). Most likely, blockade of NK_1 receptors alone is not sufficient to attenuate spinal transmission of noxious afferent input (Thompson et al., 1993). Furthermore, it seems more certain that NK_1 receptors participate in the central sensitization to noxious stimuli (Malmberg and Yaksh, 1992). In accordance with this, Jung et al. (1994) found that the highly specific and potent NK_1 antagonist SR 140333 caused complete inhibition of noxious heat-induced facilitation of thermal nociception. At the same time, the base-line responses to heat were reported to be uninfluenced by SR 140333 (Jung et al., 1994). It should be emphasized that also NK_2 receptor mechanisms have been implicated as transducers of brief thermal stimuli (Fleetwood-Walker et al., 1993; Santucci et al., 1993). Regarding mechanisms of interaction between SP and glutamate (Song and Zhao, 1994), it has been shown that SP potentiates inward glutamate-gated cation current in dorsal horn neurons (Randic et al., 1990). Furthermore, SP could possibly reduce Mg^{2+} block of NMDA receptors by activating protein kinase C (Chen and Huang, 1992).

The possible role for CGRP in central pain transmission is less obvious and more difficult to pinpoint in the absence of potent antagonists active in the CNS, but autoradiography using ^{125}I-CGRP showed distinct labeling of dorsal spinal cord (Tschopp et al., 1985; Le Grevès et al., 1989). Furthermore, CGRP potentiates the behavioral effects of noxious stimulation with SP (Wiesenfeld-Hallin et al., 1984); this was suggested to be due to interference with SP degradation (Le Grevès et al., 1989) although a postjunctional event after receptor activation also seems possible. In addition, CGRP is likely to be released from primary sensory neurons in spinal cord upon inflammation (Galeazza et al., 1995). It was recently reported that intrathecal CGRP (8–37) induced an increase in hindpaw withdrawal latency to thermal and pressure stimulation in rats, suggesting a role for CGRP in nociceptive transmission (Yu et al., 1996).

NO mechanisms are involved in central processing of primary afferent information, whereby NO is produced postsynaptically in response to, notably, NMDA receptor activation. Release of NO facilitates glutamate release via retrograde signalling or as an autocrine action (Garthwaite, 1991) (fig. 1). It is of interest that centrally administered NOS inhibitors like L-NAME exert antinociceptive actions on the response to NMDA (Meller and Gebhart, 1993). Also in the CNS there seem to be complex interactions between COX activation and NO (fig. 1). Thus, the hyperalgesia in response to both SP and NMDA receptor activation is attenuated by spinal COX inhibition (Malmberg and Yaksh, 1992).

NK_1 receptors also seem to be involved in the activation of vesicoexcitatory micturition-related reflexes in the rat spinal cord as revealed by experiments using RP67580 (Lecci et al., 1992, 1993). In contrast, the role of NMDA receptors in the spinal regulation of micturition-related reflexes is not restricted to modulation of afferent input from capsaicin-sensitive neurons but is more general (Lecci et al., 1993, Yoshiyama et al., 1993).

Also vagal sensory transmission may involve central TK mechanisms since the NK_2 antagonist SR48968 reduced citric acid-evoked cough (Advenier et al., 1993).

Activation of prejunctional receptors on central terminals of primary afferent neurons can inhibit release of both glutamate and peptide transmitters. Examples of this are α_2-agonists and morphine (acting on, for example, μ-opioid receptors) (Jessel and Iversen, 1977; Gordh, 1988; Kamisaki et al., 1993; Ueda et al., 1995a, b) (fig. 1). These agents have also been used as locally applied (intrathecal) analgesics.

G. Sensory Hyperalgesia

Hyperalgesia, i.e., lowered threshold for pain and increase in pain evoked by suprathreshold stimuli, occurs upon excitation of C-fiber afferents. Distinctions have been made between primary hyperalgesia, occurring within the area of tissue injury, and secondary hyperalgesia, occurring outside the area of injury (Lewis, 1936). Primary hyperalgesia is, at least in part, due to peripheral sensitization of nociceptive nerve endings by, for example, COX products and bradykinin (see Martin et al., 1987; Raja et al., 1988) which occurs not only in skin but also in airways (Choudry et al., 1989). After treatment with proinflammatory agents, such as Escherichia coli lipopolysaccharide, interleukin-1β or tumor necrosis factor-α, the synthesis of inducible forms of both NOS and COX (COX-2) is markedly enhanced. This induction is inhibited by antiinflammatory steroids, such as dexamethasone, but the steroids have no effect on the constitutive forms of NOS and COX (COX-1) (Moncada et al., 1991). The involvement of axon reflex mechanisms in the phenomenon of hyperalgesia has in addition been postulated (Ochoa, 1986) and, as discussed above, SP can enhance secretion of, for example, tumor necrosis factor-α (Luber-Narod et al., 1994) a factor which has been postulated to have an important role in the development of inflammatory hyperalgesia (Cunha et al., 1992). Interleukin-1β has been shown to augment the cutaneous hyperemia to capsaicin by a sensitizing action on afferent nerves, an action which involves intermedi-

ate messengers such as NO and prostaglandins (Herbert and Holzer, 1994a, b). NO may in turn activate COX to produce prostaglandins which excite sensory nerves and release peptides such as CGRP (figs. 1 and 2). Sensitization is thus expected to facilitate the neural release of TKs and CGRP, which in turn may enhance liberation of mediators from mast cells and leukocytes. Elevated synthesis of SP and CGRP accompanies peripheral inflammation (Galeazza et al., 1995). A positive feed-back loop may thus operate to cause hyperactivity of afferent nerve fibers and perpetuation of the inflammatory reaction (Lynn, 1988). The NO/COX products/sensory nerve excitation and mediator release may also be relevant in, for example, skin changes upon excessive sunburn (Deliconstantinos et al., 1995). Local capsaicin treatment of human skin causes marked long-lasting hyperalgesia to mechanical and thermal stimuli (Lundblad et al., 1987a; Simone et al., 1989). When hyperalgesia is produced by an intradermal injection of capsaicin in humans, the secondary hyperalgesia to mechanical stimulation is, however, probably due to dynamic changes in the central processing of mechano-receptive input in myelinated fibers which normally convey nonpainful tactile sensations (Torebjörk et al., 1992). The relative contribution of glutamate and NK receptor mechanisms (as well as NO and PGs) in this central "wind up" phenomenon in humans in health and disease remains to be established using selective antagonists. It is of interest that inflammation evokes increase in NK_1 receptor synthesis in intrinsic spinal cord neurons involved in nociceptive neurotransmission (Schäfer et al., 1993). This may have relevance not only for treatment of pain but also for sensory hyperreactivity in inflammation of the airways and urinary bladder, which involves a variety of peripheral and central reflex responses (figs. 1 and 2). Furthermore, the NK_1 receptor antagonist RP67580 relieves chronic hyperalgesia in diabetic rat (Courteix et al., 1993). In addition, capsaicin-sensitive bladder afferents represent possible targets for the pharmacological treatment of bladder hyperreflexia in patients with neurogenic urinary incontinence due to multiple sclerosis or spinal cord injury (Fowler et al., 1994; see Chen et al., 1995b).

H. Tachykinins and Enteric Cotransmission

Sensory nerves are abundant in the gastrointestinal tract. In cat stomach, capsaicin acts on sensory nerves to cause the release of TKs, which in turn act on a local cholinergic neuron, leading to an atropine-sensitive contraction (Delbro et al., 1983). Capsaicin also causes contraction of guinea pig ileum via TK release (Björkroth, 1981). Both SP and NKA are present not only in peripheral branches of sensory neurons innervating the gut wall but also to a major extent in local neurons, for example, in the myenteric plexus (Costa et al., 1981). Also the N-terminally extended variants of NKA, NPK and NPγ, have been identified in guinea pig intestine. NKB is largely undetectable in intestinal extracts, however (Deacon et al., 1987; Christofi et al., 1990). Similarly, the NK_3 receptor antagonist SR142801 did not influence the guinea pig ileum contraction to capsaicin (Patacchini et al., 1995). The primary source of SP-containing fibers within the muscle layers is neurons located in myenteric ganglia (Costa et al., 1981) with oral projections to, for example, circular muscle (Brookes et al., 1991). SP and NKA are colocalized in fibers supplying intestinal smooth muscle (Schmidt et al., 1991).

Circular smooth muscle often contains both NK_1 and NK_2 receptors, whereas the SP-evoked release of ACh in guinea pig myenteric neurons is mediated predominantly by NK_3 receptors (Guard et al., 1990; Guard and Watson, 1991). IP_3-induced Ca^{2+} release is likely to contribute to contraction of gastrointestinal smooth muscle by NK_1 and NK_2 receptor activation, but TKs have also been reported to modulate ionic conductances (Benham and Bolton, 1983; Nakazawa et al., 1990; Shuttleworth et al., 1993). NK_1 receptor stimulation in guinea pig colon results in activation of voltage-dependent Ca^{2+} channels, sensitive to nifedipine, whereas NK_2 receptor-evoked contractions are largely unaffected by nifedipine and are not accompanied by substantial membrane depolarization (Maggi et al., 1994c; Zagorodnyuk et al., 1994). In the guinea pig duodenum, both NK_1 and NK_2 receptors cooperate in producing NANC excitation and contraction of circular muscle. Both receptors contribute to generate action potentials via nifedipine-sensitive Ca^{2+} channels (Zagorodnyuk et al., 1995).

TKs mimic the atropine-resistant mechanical and electrical effects of nerve stimulation. In guinea pig ileum circular muscle, electrical field-stimulated contractions are mediated primarily by NK_2 receptors, although NK_1 receptors are also present (Bartho et al., 1992; Suzuki et al., 1994). Also, balloon distensions in the guinea pig ileum cause ascending excitation, which is mainly due to NK_2 receptor stimulation (Holzer et al., 1993a; Maggi et al., 1994b). In analogy with the situation for the sensory TK control of guinea pig bronchial smooth muscle, different receptors seem to be activated by nerve stimulation and by exogenous application of TKs, NK_2 receptors being preferentially activated by endogenous TKs. This may not represent a general phenomenon, however, because NK_1 receptors are likely to be involved in nerve responses of the guinea pig colon circular muscle (Zagorodnyuk et al., 1993). NK_2 receptors may also be recruited by nerve stimulation in this latter tissue during prolonged electrical field stimulation, however, suggesting specialization of NK_1 and NK_2 receptors in producing fast and slow NANC responses (Maggi et al., 1994d). Reflex stimulation experiments in vivo suggest the involvement of both NK_1 and NK_2 receptor mechanisms in the colon, again emphasizing that electrical field stimulation in vitro may release inhibi-

tory transmitters that mask the involvement of certain receptors (Giuliani et al., 1993).

There is strong evidence that TKs are colocalized with ACh in excitatory motor neurons in the intestine (Brookes et al., 1991; Llewellyn-Smith et al., 1988). Interestingly, muscarinic antagonists can abolish reflex-evoked contractions evoked by a low degree of distension, whereas TK receptor antagonists are effective only when stimulus intensity is increased (Costa et al., 1985; Grider, 1989). In contrast to the situation for TKs in primary sensory neurons, TK release from enteric motor neurons may thus require high stimulation frequencies (as for VIP and NPY in autonomic motor neurons). It is likely that TKs and ACh interact synergistically, and thus that the antagonism of one cotransmitter can reduce the effectiveness of the other (Holzer et al., 1993a). Furthermore, if a low concentration of atropine was used, the effectiveness of the NK_2 antagonist is selectively potentiated (Holzer and Maggi, 1994). Taken together, these data clearly indicate that TKs (together with ACh) are involved in excitatory transmission in the gut that can be related to the peristaltic reflex.

III. VIP/Nitric Oxide and Parasympathetic Nerves

A. VIP Peptides as Neurotransmitters

1. Synthesis, release and degradation. VIP was isolated from small intestine of the pig and the name derives from its profound and long-lasting vasodilatory action upon systemic administration (Said and Mutt, 1970a, b). Porcine VIP is a single chain linear peptide with 28-amino-acid residues and is amidated at the C-terminal (Mutt and Said, 1974). Unlike most bioactive peptides, such as TKs and NPY, the carboxyamidation of VIP is not critical for the biological activity (Fahrenkrug et al., 1989). The primary structure of VIP is related to that of PHI (peptide with N-terminal histidine and C terminal isoleucine) (in rat and pig), PHM (the counterpart of PHI in humans), and PACAP (pituitary adenylate cyclase activating peptide). PHI is generally less and PACAP is more active than VIP (Lundberg et al., 1984b; Arimura, 1992; Nilsson, 1994). VIP of mammalian origin has a conserved structure except in the guinea pig, where the peptide differs in 4-amino-acid residues. VIP is derived from a precursor, prepro-VIP, consisting of 170 amino acid residues (Itoh et al., 1983). The precursor contains both VIP and PHM/PHI in its sequence. A C-terminally extended form of PHM named PHV-42 (peptide with N-terminal histidine and C-terminal valine) can also be formed, which is just as potent as VIP in relaxing smooth muscle (Yiangou et al., 1987; Palle et al., 1992). It is clear that, although the degree of expression varies in different tissues (Fahrenkrug et al., 1985), VIP and PHI are colocalized in parasympathetic neurons around blood vessels and within visceral smooth muscle (Lundberg et al., 1984c). PACAP also seems to be present in parasympathetic nerves, at least some also containing VIP (Uddman et al., 1993), although the relation to the VIP/PHI system needs to be further clarified (Uddman et al., 1991) using, for example, in situ hybridization techniques. VIP is stored in LDV (Johansson and Lundberg, 1981; Lundberg et al., 1981e; Probert et al., 1981) and axonally transported to nerve terminals (Gilbert et al., 1980; Lundberg et al., 1981d) to replenish the terminal stores (table 3, fig. 6).

VIP release is dependent on the frequency of nerve stimulation, release being maximal at higher frequencies (around 10 Hz) (Fahrenkrug et al., 1978; Lundberg et al., 1980, 1981a, b, c; Uddman et al., 1980; Bloom and Edwards, 1980). Generally, PHI release seems to paral-

TABLE 3
Transmitter characteristics (VIP, NO, ACh)

	VIP	NO	ACh		
Synthesis	cell body precursor	NOS	CAT		
Storage	LDV	–	small clear vesicles		
Terminal resupply	axonal transport	synthesis on demand (Ca^{2+})	local synthesis		
Preferred receptor	$VIP_{(1,2)}$	–	m1	m2	m3
Selective agonist	VIP/PACAP	–	–	–	–
Antagonist	–	L-NAME (NOS)	pirenzepine	gallamine	hexahydrosiladifenidol
Second-messenger	cAMP, K^+	cGMP (PGs, $K_{Ca^{2+}}$)	IP_3,PKC	K^+, Ca^{2+}	IP_3,PKC,NO K^+
Functional response	vasodilation	vasodilation	sEPSP	inhibition of transmitter secretion	vasodilation
	smooth muscle relaxation exocrine secretion	smooth muscle relaxation exocrine secretion	HCl secretion	bradycardia	smooth muscle contraction exocrine secretion
Removal	peptidase (NEP)	superoxide Hb	AChE	–	–

Summary of transmitter characteristics for VIP, NO and ACh, which coexist in postganglionic parasympathetic neurons.

NOS, neuronal nitric oxide synthase; CAT, choline acetyltransferase; LDV, large dense-cored vesicles; PACAP, pituitary adenylate cyclase activating peptide; IP_3, inositoltrisphosphate; PKC, protein kinase C; AChE, acetylcholine esterase.

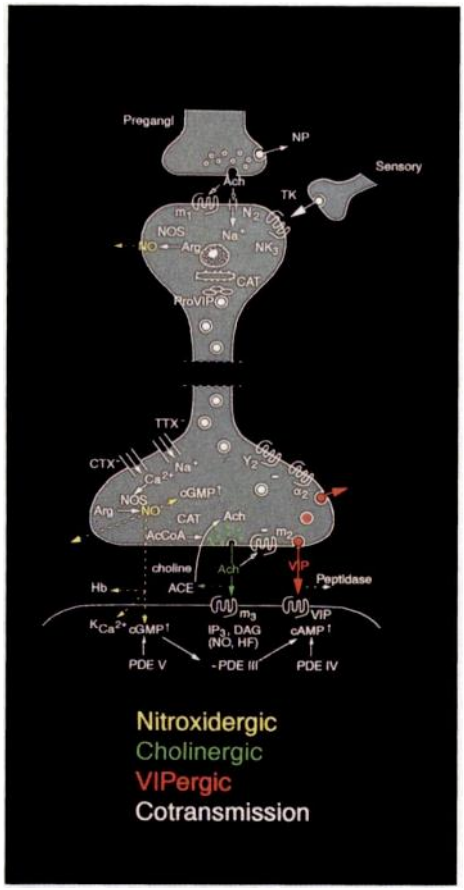

FIG. 6. Schematic illustration of a parasympathetic neuron containg synthesis machinery to produce a gas, NO, a classical transmitter acetylcholine (Ach), and peptides of the VIP family. A preganglionic cholinergic terminal and an axon collateral from a capsaicin-sensitive sensory neuron have established contacts with this multitransmitter postganglionic neuron. NO is produced from L-arginine by NOS upon elevation of intracellular Ca^{2+} in the cell body or terminal (due to, for example, TTX-sensitive depolarization and influx through ω-conotoxin (CTX)-sensitive N-type Ca^{2+} channels). Subsequently, NO may either diffuse directly or bind to some carrier thiol groups, initially influencing the same neuron and subsequently the adjacent structures. Guanylyl cyclase is activated by NO, leading to formation of cGMP. Activation of the cGMP system leads to vasodilation, relaxation of nonvascular smooth muscle, exocrine secretion and enhancement of transmitter release. In addition, NO itself can open Ca^{2+}-dependent K^+ channels, $K_{Ca}{}^{2+}$, apparently without requiring cGMP formation. NO is rapidly inactivated, for example, by binding to proteins containing heme groups, such as hemoglobin (Hb). Ach is synthesized locally by choline acetyltransferase (CAT) and prestored in small, clear vesicles. Upon depolarization, Ach is released and acts on muscarinic receptors on target cells (m3 on blood vessels and certain exocrine glands) and prejunctionally (m2), inhibiting transmitter release. After Ach destruction by acetylcholine esterase (ACE), choline is recycled for Ach resynthesis. VIP (and the related peptides PHI and PACAP) are synthesized as precursors in the cell body, stored in LDV, processed and axonally transported to release sites in terminals. Depolarization leads to VIP release and the peptide binds to G-protein-coupled receptors linked to cAMP formation and functional responses such as vasodilation, exocrine secretion and relaxation of nonvascular smooth muscle. VIP is removed from release sites at least partly by diffusion and also by peptidases. At the second-messenger level, a variety of interactions may occur, especially regarding PDE regulation of cAMP and cGMP levels (PDE IV preferentially degrades cAMP and PDE V cGMP. PDE III is a cGMP-regulated cAMP PDE). Various specific inhibitors for PDE isoforms have been developed: milrinone for PDE III, rolipram for PDE IV and zaprinast for PDE V. Atropine blocks nerve-evoked vasodilation upon low frequency stimulation and blocks exocrine secretion in salivary glands. In pancreas, most of the parasympathetic secretion and vasodilation is atropine-resistant but is inhibited by VIP antiserum. NOS inhibition attenuates the parasympathetic vasodilatory response in salivary glands but not in pancreas, where, however, NOS inhibition has clear-cut effects on the exocrine secretion response. Prejunctional α_2-adrenergic and Y_2-ergic receptors may inhibit transmitter release from these neurons when NA and NPY, released from adjacent sympathetic nerves, have reached appropriate receptors by diffusion. At the cell body level, primary cholinergic ganglionic transmission involves nicotinic-2 (N_2) receptors with some contribution by facilitatory m1 receptors. Furthermore, tachykinins such as SP released from sensory axon collaterals may also facilitate ganglionic transmission via NK_3 (or NK_1) receptor-mediated slow EPSP. In addition, preganglionic nerves contain LDV with neuropeptides (NPs) such as endogenous opioids.

lel that of VIP (Lundberg et al., 1984d; Fahrenkrug, 1987; Holst et al., 1987). VIP has a relatively long half-life (several minutes) (Domsche et al., 1978). The biological response is terminated mainly by enzymatic degradation of the peptide after its binding to the receptor, and possibly a small fraction is internalized into the target cells (Luis et al., 1988). Neutral endopeptidase (NEP24.11) can readily degrade VIP (Goetzl et al., 1989).

2. Biological actions. VIP is a powerful vasodilator in most vascular beds (Fahrenkrug, 1993), and it also has a positive chronotropic and inotropic effect on the heart in various species, including humans (Franco-Cereceda et al., 1987e). The vascular VIP effect is generally considered to be independent of the endothelium (Pernow, 1989) and, with some exceptions, to be exerted directly onto vascular smooth muscle (Itoh et al., 1985; Ganz et al., 1986). VIP is also a relaxant of visceral smooth muscle in the digestive, urogenital, and respiratory systems. Furthermore, VIP stimulates exocrine secretion from the pancreas and intestine (Fahrenkrug, 1993) and enhances salivary secretion (volume and protein content) evoked by ACh (Lundberg et al., 1980, 1982a; Ekström and Tobin, 1990). PHI is generally less biologically active than VIP (Lundberg et al., 1984c). Both PACAP-27 and PACAP-38 are approximately 100 times more potent than VIP and PHI as vasodilators in the rabbit (Nilsson, 1994). In humans and several other species, however, the difference in potency between PACAP and VIP is less (see Nilsson, 1994). VIP also exerts trophic actions on growth of human keratinocytes (Hägerstrand et al., 1989).

3. VIP receptors. Two types of VIP receptors have been cloned, designated VIP1 (Ishihara et al., 1992) and VIP2 (Lutz et al., 1993) (table 3). These membrane-bound receptors have seven transmembrane domains and are G-protein coupled as are other neuropeptide receptors (see above and below). VIP receptor stimulation has been shown to elevate cellular cAMP levels (Amiranoff

and Rosselin, 1982; Schoeffter and Stoclet, 1985) through a GTP-regulated coupling and a Gs protein (Couvineau et al., 1990) (table 3). The cAMP-stimulating effect of VIP, at least in the submandibular gland, is potentiated by the muscarinic receptor agonist carbachol (Enyedi et al., 1982; Fredholm and Lundberg, 1982). In addition to adenylate cyclase, VIP receptors may be linked to alternate signal transduction systems, including inositolphosphate in sympathetic ganglia (Audigier et al., 1986). Elements within the entire primary sequence of the VIP molecule appear to be necessary for recognition by VIP receptors (O'Donnell et al., 1991). Two classes of VIP receptors with different affinity and specificity have been described (Luis et al., 1989; Robberecht et al., 1990). PHI and PACAP also interact with these receptors. PACAP usually binds with much higher affinity than PHI; however, the relative potency of these peptides at the VIP receptor in different tissues and species remains to be further established (Arimura, 1992). Separate PACAP receptors may also exist that are coupled to apamin-sensitive K^+ channels (Jin et al., 1994).

4. VIP receptor antagonists and VIP-ergic transmission. In the absence of potent nonpeptide receptor antagonists, antiserum directed against VIP has been used to inhibit parasympathetic NANC responses, including the parasympathetic nerve-mediated vasodilation in the submandibular gland (Lundberg et al., 1981c), maintenance of nerve stimulation-evoked penile erection (Jennermann et al., 1987), pancreatic bicarbonate secretion by vagal stimulation (Holst et al., 1984), reflex relaxation of the stomach (Bojo et al., 1993), nerve stimulation-evoked relaxation of trachea (Matsuzaki et al., 1980) and esophageal sphincter (Biancani et al., 1984). Therefore, VIP antisera seem to be able to neutralize the neuronally released VIP, thus leading to an inhibition of the nerve-mediated NANC responses. Bronchodilation induced by exogenous VIP in vitro is potentiated by inhibition of NEP, although the NANC relaxation-evoked nerve stimulation is not (Farmer and Togo, 1990). It should be emphasized that residual functional NANC responses are present after VIP antiserum pretreatment and that the effects of PHI and PACAP may still be in operation, as may those of NO. A VIP analogue (Lys^1, $Pro^{2,5}$, $Arg^{3,4}$, Tyr^6) VIP was recently reported to inhibit both the VIP and PACAP-38-induced increase in vertebral artery blood flow (Seki et al., 1995), but data on the influence of this antagonist on parasympathetic NANC responses remains to be established.

5. VIP and pathophysiology. A lack of VIP fibers has been implied in impotence (Gu et al., 1984), and achalasia of the esophagus (Aggestrup et al., 1983), i.e., conditions in which smooth muscle relaxation is impaired. Local administration of VIP agonists therefore has some potential in, for example, impotence (Gerstenberg et al., 1992) and possibly asthma. Because the C-terminal amidation is not necessary for biological activity, mass production of VIP agonists may also be more easily accomplished by bacterial large scale cloning techniques, although general drawbacks of peptides, such as poor oral availability and metabolic instability, clearly favor small nonpeptide peptidomimetics (and antagonists). Enhanced VIP production is seen in certain human tumors, VIPomas, which are associated with, for example, severe diarrhea and hypotension. VIP antagonists may also prove to be of value to reduce swelling and congestion of the nasal mucosa in patients with vasomotor rhinitis, a condition with hyperreactive parasympathetic reflexes (Stjärne, 1989a). Furthermore, VIP may be released in nasal allergy (Chaen et al., 1993).

B. Nitric Oxide as Neurotransmitter

1. Synthesis, release and degradation. NO is an unconventional transmitter that is not prestored and packaged in vesicles but rather is produced on demand (depolarization) and then quickly diffuses from its site of production in a random direction, being extremely membrane permeant (fig. 6; table 3). The idea that NO may participate in modulating neuronal function arose from the discovery by Furchgott and Zawadski (1980), that ACh-induced relaxation of rabbit aorta required the presence of endothelial cells. This principle also holds for other mediators, such as SP and bradykinin (Furchgott, 1984). A diffusible short-lived factor produced in endothelial cells, EDRF was proposed to account for the observed smooth muscle relaxation. The endothelium-dependent relaxation produced by ACh was Ca^{2+}-dependent (Griffith et al., 1986) and thought to be mediated by rises in cGMP in the muscle (Rapoport et al., 1983). Several nitrovasodilators known to generate NO (e.g., nitroglycerine and sodium nitroprusside) did not require an intact endothelium to elicit relaxation. Thus, it was proposed that EDRF was NO: (*a*) both agents are extremely labile (half-life a few seconds), (*b*) the relaxations evoked by both substances are blocked by hemoglobin (which binds NO) or by generation of oxygen radicals, and (*c*) the effects of both NO and EDRF are enhanced by superoxide dismutase, which scavenges superoxide ions. Subsequently, it was directly demonstrated that the vascular endothelium actually releases NO in quantities sufficient to account for the biological activity of EDRF (Ignarro et al., 1987a, b; Palmer et al., 1987). The first clear demonstration that NO acts as a neuronal messenger (on activation of NMDA receptors) came from studies on the cerebellum (Garthwaite et al., 1988, see Garthwaite, 1991).

Endogenous NO is produced from L-arginine by nitric oxide synthase (NOS), resulting in the stoichiometric production of L-citrulline (Mayer et al., 1989; Palmer and Moncada, 1989). There are several forms of NOS. An inducible form of NOS is present in macrophages which, upon activation, can produce very large amounts of NO. The constitutive NOS exists in endothelial cells (Mayer et al., 1989; Busse and Mülsch, 1990) and neuronal cells

(Bredt et al., 1990). This enzyme is Ca^{2+}-, calmodulin-, and NADPH-dependent (Palmer and Moncada, 1989). The presence of NOS in both the central (Bredt et al., 1990) and the peripheral nervous system—for example, in postganglionic parasympathetic nerves (Ceccatelli et al., 1992; Kummer et al., 1992) and preganglionic sympathetic nerves (Blottner and Baumgarten, 1992)—supported the functional evidence that NO could serve as a NANC inhibitory transmitter (Gillespie et al., 1989, 1990). Upon neuronal depolarization, intracellular Ca^{2+} is elevated, which, together with calmodulin, leads to activation of NOS and thus to NO production (Knowles et al., 1989; Bredt and Snyder, 1990). It should be emphasized that the primary source of Ca^{2+} may be inositoltrisphosphate-mediated release from intracellular stores (as for ACh, bradykinin or SP) or flux of Ca^{2+} through voltage-sensitive Ca^{2+} channels, such as N-type channels. It is of interest that the N-Ca^{2+} channel blocker CTX can inhibit the NANC tracheal relaxation, which seems to involve NO as one messenger (Altiere et al., 1992) (fig. 6).

Although the mediator of nitroxidergic transmission most likely is closely related to NO, its precise chemical nature still remains uncertain and could also represent a NO-yielding compound such as a nitrosothiol (Knudsen et al., 1992; Rand and Li, 1993), which may serve as a carrier molecule. It is thus possible that after activation of NOS, the generated NO may bind to thiol-containing amino acids, which serve as carriers; in that case, NOS inhibitors, but not the scavenger Hb, have effects on the neurogenic relaxant responses (Jenkinson et al., 1995).

2. Biological actions. NO donors such as nitroglycerine and nitroprusside have long been used in clinical practice as vasodilatory agents upon systemic administration. In organ bath experiments, it has been shown that, although NO is very labile, it relaxes visceral smooth muscle in the respiratory, urogenital and digestive tracts (Gillespie et al., 1990). Inhaled NO exerts potent local vasodilatory effects in the bronchial mucosa (Alving et al., 1993) and pulmonary circulation (Frostell et al., 1993) without systemic vascular actions, presumably due to rapid binding to Hb in red blood cells (Martin et al., 1985). It is likely that the powerful vasodilatory effects of cigarette smoke inhalation in the airways are caused by the NO component in the smoke (Alving et al., 1993).

3. Nitric oxide-second-messenger system. The major effector of NO identified in many tissues is soluble guanylyl cyclase (Arnold et al., 1977; Murad et al., 1978). NO binds tightly to the heme region of the cyclase, leading to a conformational change and enzyme activation (Wolin et al., 1982). The resulting rises in cGMP levels can then affect ion channel or PDE activity (such as cGMP regulated cAMP PDE, i.e., PDE III, Rascon et al., 1992) (fig. 6) or activate a cGMP-dependent protein kinase. In smooth muscle cells, the NO-induced rises in cGMP may activate a cGMP-dependent protein kinase that is ultimately responsible for relaxation (Rapoport et al., 1983). Alternatively, cGMP can decrease intracellular Ca^{2+} levels, which also can contribute to relaxation (Rashatwar et al., 1987; Waldman and Murad, 1987). The levels of cGMP are regulated by PDE V.

NO can also act in cGMP-independent ways. For example, the cytotoxic action of macrophage-derived NO on tumor cells and other tissue components is the result of nitrosylation and subsequent inactivation of iron and iron-containing enzymes of the respiratory cycle and DNA synthesis (Nathan, 1992). Furthermore, NO may directly interact with COX to cause an increase in the enzymatic activity leading to formation of various PGs (Salvemini et al., 1993), presumably, again, by interacting with the iron-heme center at the active site. The reaction of NO with superoxide anions leads to a cytotoxic oxidant, peroxynitrite (Beckman et al., 1990), which may contribute to tissue injury in a number of pathophysiological situations, such as inflammation, ischemia-reperfusion (Downey, 1990) and brain ischemia (Lipton et al., 1993).

4. Nitric oxide antagonists. Initially, Hb was used as a scavenger for NO, which binds avidly to iron in the heme group. Because Hb is a large protein, it is unlikely to cross cellular membranes. Thus, when applied extracellularly, Hb is considered to provide information regarding the role of NO as an *intercellular* rather than *intracellular* messenger. It should be emphasized that the capacity for NO neutralization is likely to be different in vivo than in vitro due to a continuous resupply of Hb in circulating red blood cells. Furthermore, in the in vitro studies, large amounts of Hb is applied in the extravascular compartments, which is less likely to occur under normal in vivo conditions. Superoxide anions can also neutralize NO. It must also be borne in mind that the effect of Hb on neuronal mediators is not limited to NO binding, since Hb, being a large protein, may also in high concentrations to some extent influence peptides (Hemsén and Lundberg, 1992) including VIP (Hemsén and Lundberg, unpublished).

Several competitive inhibitors of the NOS are available and have been widely used as pharmacological antagonists of NO production. Thus, L-arginine analogs substituted at the guanidino nitrogens act as false substrates, thereby blocking NOS activity. Examples of this are N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine (L-NNA) and L-nitro-arginine-methyl-ester (L-NAME) (table 3). Many of these compounds also have D-isomeric forms that do not inhibit NOS and thus serve as useful controls. L-arginine in excess, classically reverses the effects of these NOS inhibitors on NO production (Rees et al., 1989), although it is notoriously difficult to reverse the effects of high doses of L-NNA under in vivo or in vitro conditions (Holmquist et al., 1991; Holst et al., 1994; Modin et al., 1994a). Some of these agents, like L-NAME, also interact with muscarinic re-

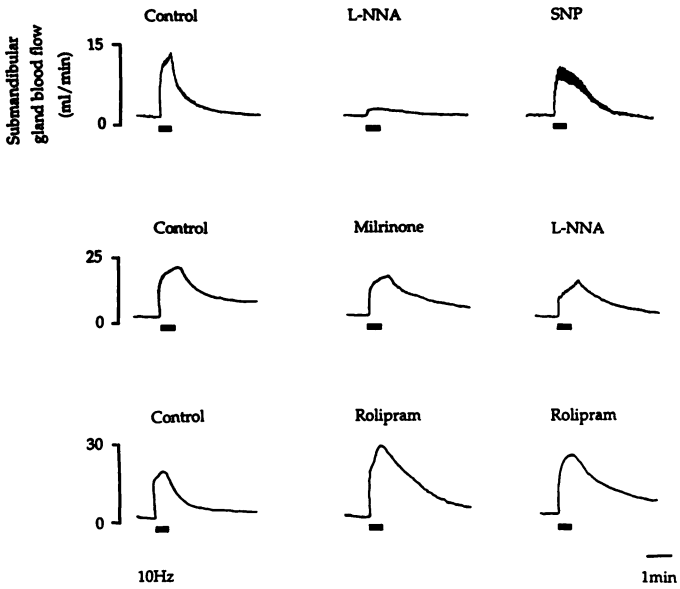

FIG. 7. Recordings of in vivo vasodilatory responses in pig submandibular gland on parasympathetic nerve stimulation before and after various pretreatments. *Top panel*: reversal by sodium nitroprusside (SNP) (0.5 μg/kg/min i.v.) of the inhibitory effects of the NOS inhibitor L-NNA (50 mg/kg i.v. and 5 mg/kg/min infusion) on parasympathetic vasodilation (electrical stimulation, 10 Hz for 30 seconds). *Middle panel*: the PDE III inhibitor milrinone (50 μg/kg i.v. and 5 μg/kg/min infusion) also partially prevents the L-NNA inhibition of parasympathetic vasodilation (modified from Modin et al., 1994b). *Lower panel*: the PDE IV inhibitor rolipram (200 μg/kg i.v.) enhances, in contrast to milrinone, the parasympathetic vasodilation response in the absence of L-NNA. Time scale is 1 minute.

ceptor mechanisms, however, as revealed by evoking atropine-sensitive salivary secretion in vivo or displacing muscarinic receptor binding in vitro. (Edwards and Garret, 1992, 1993; Buxton et al., 1993). Unfortunately, these NOS inhibitors do not readily distinguish between endothelial and neuronal NOS isoforms, which complicates interpretation of data, especially in vivo. Thus, in vivo, NOS inhibition leads to dose-dependent vasoconstriction with decreased local energy supply and changes in basal tone of vascular smooth muscle (Rees et al., 1989; Gardiner et al., 1990b; Modin et al., 1994a). This may in itself impair the neurotransmission process. Most likely, basal NO production by the constitutive endothelial NOS is stimulated by shear stress forces and acts as a major regulatory mechanism for basal vascular tone (Moncada et al., 1991; Vargas et al., 1990, 1991). Sympathetic neuroeffector mechanisms also seem to influence NO production because the vascular effects of NOS inhibition are reduced after sympathetic denervation or ganglionic blockade (Vargas et al., 1990; Lacolley et al., 1991). This effect may, however, be indirectly due to changed shear stress forces after loss of sympathetic vasoconstrictor tone.

5. Nitroxidergic transmission. Studies conducted in a variety of preparations and tissues, mainly using in vitro conditions, have suggested the NANC relaxation of visceral smooth muscle evoked by electrical field stimulation depends on NO, released as a transmitter from parasympathetic terminals (Gillespie et al., 1990). The NO dependency was revealed by the use of Hb and NOS inhibitors in studies of, for example, the rat anococcygeus muscle (Gillespie et al., 1989; Li and Rand, 1989; Gibson et al., 1989). Furthermore, NO has been postulated to mediate at least part of the NANC neuronal relaxation of tracheobronchial (Tucker et al., 1990; Belvisi et al., 1991), gastrointestinal (Maklouf and Grider, 1993), and urethral smooth muscle (Persson and Andersson, 1994). When L-NAME was administred in vivo, decreased micturition volume and bladder capacity and increased spontaneous bladder contractions were observed (Persson et al., 1992). NO was also suggested to mediate the noncholinergic vasodilatory response to nerve stimulation in cerebral and mesenteric vessels (Toda and Okamura, 1990a,b), nasal mucosa (Watanabe et al., 1995) as well as penile erection (Holmquist et al., 1991; Burnett et al., 1992; Rajfer et al., 1992). Holst et al. (1994) concluded that formation of NO was essential for vagus nerve-evoked pancreatic exocrine secretion and basal vascular tone, but not for vagus-evoked NANC vasodilation. NO mechanisms also seems to be involved in the vasodilatory response to parasympathetic stimulation of the submandibular salivary gland (Edwards and Garret, 1993; Modin et al., 1994b, c) (fig. 7). In this context, it is interesting that NOS gene knock-out mice, lacking NOS in peripheral NANC nerves, were viable and even fertile, indications that other factors, such as VIP, are also of relevance for, for example, penile erection (Huang et al., 1993). However, the gene knock-out technique has the drawback of allowing introduction of compensatory mechanisms during development.

Inhibition of NO production by L-arginine analogs has also been shown to attenuate the postjunctional vascular responses to other agents that are considered to act independently of the endothelium (Thomas et al., 1989). Furthermore, the vasodilatory response to exogenous VIP was also reduced by NOS inhibitors (Gaw et al., 1991; Edwards and Garrett, 1993; Modin et al., 1994a). In addition, the exocrine fluid secretion in response to VIP in the pancreas was reduced by both L-NAME and L-NNA (Holst et al., 1994). It is of interest that cGMP and/or NO may be involved in the second-messenger system for VIP in addition to the cAMP pathway (Maklouf and Grider, 1993). Thus, the levels of cAMP and cGMP are regulated by PDEs that are present in various forms, including cGMP regulated cAMP PDE (PDE III), the cAMP-specific PDE (PDE IV) and the cGMP-specific PDE (PDE V) (Murray and England, 1992). Therefore, NO/cGMP may indirectly enhance the effects of VIP, whereas NOS inhibitors may reduce the effects of VIP by increasing cAMP degradation (fig. 7).

NO modulates Ca^{2+} channel currents in sympathetic neurons (Chen and Schofield, 1993). L-NNA can also modulate the cGMP content in rat superior cervical sympathetic ganglion upon preganglionic nerve stimulation

(Sheng et al., 1992), which is of interest considering the presence of NOS in preganglionic sympathetic cholinergic neurons. Preliminary data (Modin et al., 1994b) do not show that NO plays any major role as preganglionic transmitter in the control of postganglionic sympathetic vasoconstrictor neurons in vivo, however, although it should be emphasized again that the NOS inhibitors cause marked changes in basal vascular tone, which complicates the interpretation of experimental data obtained in vivo.

6. Nitric oxide and pathophysiology. Loss of NOS nerves has been reported in esophageal achalasia (Mearin et al., 1993) and infantile hypertrophic pyloric stenosis (Vanderwinden et al., 1992). Interestingly, a similar phenomenon with development of grossly enlarged stomach with hypertrophy of the pyloric sphincter and the circular muscle layer is observed after disrupting the neuronal NOS gene (Huang et al., 1993). Local injection of NO donors can induce penile erection and may be used in impotence (Andersson, 1993). After damage of the peripheral axons of sensory neurons, a marked up-regulation of the NOS expression in primary sensory neurons occurs in spinal ganglia but not in vagal ganglia (see Hökfelt et al., 1994). Because NOS-derived NO has been suggested to regulate release of peptide transmitters from sensory neurons (see Holzer and Jocic, 1994), this indicates potential facilitation of certain release processes (see above under section II.G., Sensory Hyperalgesia).

C. VIP/Nitric Oxide and Acetylcholine Cotransmission

1. Prejunctional interactions. Peptide release (VIP and PHI) from parasympathetic cholinergic nerves in cat submandibular salivary gland seems to be regulated by prejunctional muscarinic receptors (presumably of m2 type): after atropine treatment, there are parallel increases in the nerve stimulation-evoked outflow of these peptides (Lundberg et al., 1981a, c, 1984d). Recent data suggest that L-NNA in high doses suppresses peptide overflow from parasympathetic nerves in submandibular salivary gland (Modin et al., 1994a), pancreas (Holst et al., 1994) and intestine (Grider et al., 1992; Maklouf and Grider, 1993). In several brain regions, NO modulates synaptic function by altering the release of transmitter from presynaptic nerve endings. Thus, in cholinergic neurons also containing NOS, it was found that an NO donor enhanced basal release of ACh. Furthermore, NOS inhibition reduced basal release of ACh, suggesting that there is a continuous NO production that regulates transmitter release from these neurons (Prast and Phillipu, 1992). Other studies also indicate that NO or cGMP enhances ACh release (Sandberg et al., 1989; Hirsch et al., 1993). NO has been reported to modulate N-type Ca^{2+} channels (Lonart et al., 1992; Chen and Schofield, 1993), which are likely to be involved in transmitter secretion, including release of neuropeptides from LDV (Haas et al., 1989a, b, 1990; Lou et al., 1992a). Alternatively, NO production, which elevates cGMP levels, may lead to activation of cGMP-dependent protein kinases, which augment the phosphorylation of proteins that are involved in exocytotic mechanisms of neurotransmitter release (Greengard et al., 1993). Thus, there may be a connection between NOS activity and NO production and exocytosis of transmitters (both ACh and peptides) (fig. 6). Indeed, at least in one tissue where peptide overflow upon parasympathetic stimulation had been attenuated by the NOS inhibitor L-NNA, the inhibition was reversed by local production of NO from nitroprusside (Modin et al., 1994b) (fig. 7).

The reduction in transmitter (VIP) outflow after NOS inhibition may thus, at least partially, contribute to the suppressed vasodilatory response to parasympathetic nerve stimulation and should be taken into consideration when establishing the role of NO in NANC transmission.

2. Postjunctional interactions. In parasympathetic vascular control, the vasodilator response to nerve stimulation at low frequencies or a moderate number of impulses in salivary glands of cat and pig is atropine-sensitive, illustrating that the effect is mediated by ACh acting on muscarinic receptors (Darke and Smaje, 1972; Lundberg et al., 1981c; Modin et al., 1994b), presumably of the m3 type. These responses are also similar to those evoked by exogenous ACh, being rapid in onset and short-lasting as well as atropine-sensitive. This vasodilatory response to low frequency stimulation is markedly reduced by NOS inhibition (Modin et al., 1994b), in agreement with the view that endothelially derived NO is the primary mediator for ACh-evoked vasodilation (Furchgott and Zawadski, 1980). In the precontracted cat pulmonary vascular bed, vagal stimulation elicits a relaxation that is blocked by atropine and greatly inhibited by L-NAME and by attenuation of guanylyl cyclase activity. By contrast, L-NAME has no inhibitory effects on the dilator response to, for example, the NO donor sodium nitroprusside (McMahon et al., 1992). Incomplete inhibition of ACh-evoked vasodilation by NOS inhibitors has also been reported in vivo (Mügge et al., 1991; Modin et al., 1994a) and in vitro (Garland and McPherson, 1992). The vascular activity of ACh is apparently more NO-dependent in large than in small coronary arterioles (Komaru et al., 1991). This apparent anomaly has been suggested to be related to ACh-evoked release of some factor with hyperpolarizing actions other than NO from the endothelium opening a K^+ channel (Garland and McPherson, 1992; Nagao and Vanhoutte, 1993). Interestingly, NO itself can open Ca^{2+}-dependent K^+ channels and cause hyperpolarization (Tare et al., 1990), apparently without requiring cGMP formation (Bolotina et al., 1994) (table 3). Additionally, charybdotoxin, a specific inhibitor of large conductance, Ca^{2+} activated K^+ channels, abolishes the methylene blue resistant component in the NO-induced relaxation of rabbit aorta (Bolotina et al., 1994). Because the PDE III

inhibitor milrinone, which elevates cAMP, partly prevented the inhibitory effect of L-NNA on the submandibular salivary gland vasodilation in response to exogenous ACh or to the endogenous, atropine-sensitive parasympathetic component, the muscarinic second-messenger system for ACh may thus be at least partly independent of the NO/cGMP pathway (Modin et al., 1994b). In analogy, for the situation with sensory nerve-evoked vasodilation, it seems surprising and less likely that in vivo, a very enzymatically labile transmitter, in this case ACh, first should diffuse from release sites at the adventitiomedial junction through the arteriolar smooth muscle layer before activating appropriate receptors on endothelial cells (Lundberg et al., 1982b).

The atropine-resistant component of parasympathetic vasodilation in salivary glands is markedly suppressed by NOS inhibition (Edwards and Garret, 1993; Modin et al., 1994b, c) (fig. 7), suggesting neuronal NO as mediator in noncholinergic vascular control as well. However, the vasodilating effects of the NO donor nitroprusside were short-lasting, in agreement with the short half-life of NO (a few seconds), compared with the long-lasting NANC response to parasympathetic nerve stimulation, which more resembles the effect of VIP (which has a half-life of several minutes). As discussed above, L-NNA may reduce both peptide release and the vasodilatory effect of VIP. Normally, basal production of NO causes elevated levels of cGMP, which prevents cAMP breakdown via PDE III (Rascón et al., 1992). Because the PDE III inhibitor milrinone attenuates the L-NNA-induced reduction of the parasympathetic nerve stimulation and VIP responses and the NO donor nitroprusside restored the effects after L-NNA (fig. 7), this clearly suggests that NO release may be crucial for an optimal VIP effect (Modin et al., 1994b, c). This effect is seen mainly after NOS inhibition: under normal conditions nitroprusside or milrinone do not enhance the VIP or parasympathetic nerve stimulation responses (fig. 7). In pig submandibular gland, the PDE IV inhibitor rolipram, on the other hand, enhances the vasodilatory response to parasympathetic nerve stimulation (fig. 7), as well as the effect of VIP, suggesting that cAMP degradation by PDE IV is more crucial for these effects than PDE III under control conditions. Because PDE III is inhibited by cGMP, the NANC transmitter or NO donors may in turn be acting as PDE III inhibitors, not only in vascular smooth muscle but also in other tissues. The increase in cellular cAMP in human bronchi caused by this inhibition of PDE III is normally slight because of the presence of PDE IV. When PDE IV is inhibited, however, this increase in cAMP by PDE III blockade is elevated and relaxation potentiated (Torphy et al., 1993). Similar data have earlier been obtained in vascular smooth muscle (Lindgren et al., 1991).

Electrical field stimulation causes urethral relaxation and increase in tissue cGMP content. Both these responses are potentiated by the PDE V inhibitor zaprinast and inhibited by L-NNA, suggesting the importance of the NO-cGMP pathway (Persson and Andersson, 1994). These findings are in agreement with data from electrical field stimulation-evoked contractions of guinea pig pulmonary artery in vitro where zaprinast enhances the NANC relaxant response, which also is characterized by a L-arginine reversible L-NAME inhibition (Liu et al., 1992b). Inhibition of the cAMP-specific PDE (PDE IV) by rolipram enhances the NANC relaxation in guinea pig bronchi, indicating the importance of a cAMP-producing mediator (such as VIP) (Undem et al., 1994). Rolipram also potentiates NANC relaxation of human bronchi, whereas zaprinast had only a minor enhancement effect in spite of marked inhibition by L-NNA (Fernandes et al., 1994). Furthermore, rolipram also potentiated relaxant responses to NO donors, suggesting that cAMP plays a facilatory role in NANC relaxation of human bronchi.

Taken together, these data mainly using the parasympathetic neuronal control of blood flow in the submandibular gland as an in vivo model, but also in isolated tissues in vitro, have revealed interesting preganglionic and postganglionic interactions in this multimessenger system involving a classical transmitter, ACh, peptides such as VIP, and the gas NO. In this complex system, it is possible that drugs that are meant to inhibit muscarinic receptors or NO production also have prejunctional effects on peptide NO and ACh release and that the net effect is opposite to that intended. Furthermore, the NO/cGMP pathway seems to have a regulatory role also at postjunctional sites, especially for the cAMP system (fig. 6). It is striking that there seems to be clear-cut tissue and species variations regarding the relative contribution by ACh, VIP and NO mechanisms for parasympathetic functional responses. Thus, in some cases (salivary glands), cholinergic mechanisms (and probably VIP-like peptides and NO) contribute to neurogenic relaxation of vascular smooth muscle (Lundberg, 1981; Edwards and Garret, 1993; Modin et al., 1994b, c). In other cases (pig pancreas) (Holst et al., 1994), VIP-like peptides may be the most important mediator for vagal vasodilation. On the other hand, in cerebral vessels and nasal mucosa in vitro, NO may be the sole mediator for field stimulation-evoked neurogenic relaxation (Toda and Okamura, 1990b; Watanabe et al., 1995). Regarding other types of relaxant responses, the inhibitory NANC transmission in, for example, rat gastric fundus or guinea pig airways (Tucker et al., 1990) is likely to be mediated by both NO and VIP (Li and Rand, 1990; D'Amato et al., 1992), whereas the primary NANC transmitter in the rat anococcygeus muscle or pig urethra is NO or a closely related compound (Li and Rand, 1989; Gillespie et al., 1989; Andersson, 1993). In addition to NO, another relaxant factor with much more long-lasting actions is released, especially at high stimulation frequencies in the pig urethra (Werkström et al., 1995). It is tempting to speculate that this could be due

to VIP or a related peptide. For final characterization of the parasympathetic, NANC mechanisms, it will be necessary to develop nonpeptide receptor antagonists for various peptides of the VIP/PACAP family in addition to selective inhibitors of the neuronal form of NOS.

D. VIP/Nitric Oxide and Enteric Cotransmission

VIP and PHI are found in, for example, enteric neurons that project anally to the circular smooth muscle layer, an anatomical arrangement compatible with neurons that are involved in the descending inhibitory component of the peristaltic reflex (Yanihara et al., 1983; Furness et al., 1992b; Bredkjaer et al., 1994). There may be differences in the posttranslational processing of these peptides in different enteric neuronal populations (Raybould and Dimaline, 1987; Fahrenkrug, 1993). Also PACAP 27 and PACAP 38 have been identified in gut extracts (Arimura et al., 1991), and VIP and PACAP seem to coexist in enteric neurons, at least in some species (Sundler et al., 1992).

As discussed above, multiple receptor subtypes have been identified for the VIP family of peptides, and VIP receptor classification is based on the relative activities of natural ligands because potent nonpeptide receptor antagonists are not yet available (Harmar and Lutz, 1994). VIP receptors bind PACAP 27 and 38 as well as VIP with similar affinities and are coupled to stimulation of adenylyl cyclase. The cloned VIP1 receptor seems to dominate in the gastrointestinal tract (Harmar and Lutz, 1994). The PACAP receptor has low affinity for VIP and may be unique in being coupled to apamin-sensitive K^+ channels in gastrointestinal smooth muscle. Relaxation with PACAP 27 and 38 were both antagonized by the fragment PACAP(6–38) (Jin et al., 1994; Schworer et al., 1992).

A variety of observations suggest that VIP, PHI and PACAP are involved as inhibitory transmitter substances in the gastrointestinal tract (Fahrenkrug, 1993; Jin et al., 1994). Fast, apamin-sensitive inhibitory junction potentials (IJPs) have been recorded from a number of preparations, with a more slowly developing IJP superimposed upon the fast event. This slower IJP is blocked by NOS inhibitors (Lyster et al., 1992; He and Goyal, 1993; Stark et al., 1993). ATP has previously been considered a major mediator candidate for the apamin-sensitive rapid component of neural inhibition (Shuba and Vladimirova, 1980) and in the guinea pig duodenum suramin (a P_{2x} and P_{2y} antagonist) blocks the fast phase of the IJP (Zagorodnyuk et al., 1995). Recently, it was reported that PACAP antisera and a PACAP receptor antagonist also block the apamin-sensitive component of nerve-evoked relaxation in guinea pig taenia coli, suggesting involvement of PACAP in this response (Jin et al., 1994).

VIP may cause hyperpolarization indirectly via the release of NO from smooth muscle (He and Goyal, 1993; Murthy and Makhlouf, 1994) or serve as an inhibitory transmitter without generating a distinct IJP. Data from studies using immunoneutralization favor VIP as a transmitter in the stomach (Bojo et al., 1993; Lefebre, 1993). As discussed above, NOS is coexpressed with VIP in enteric neurons (Furness et al., 1992a, b). It is therefore likely that VIP-like peptides and NO act in concert to produce inhibition of intestinal contraction (see Keef et al., 1994). Blockade of NOS reduces the threshold for initiation of the intestinal peristaltic contraction (Waterman and Costa, 1994). In the rat gastric fundus, nitroxiotergic transmission is predominant at lower frequencies, whereas the VIP component requires higher stimulation frequency (Li and Rand, 1990). Finally, NOS inhibition using L-NNA has been reported to inhibit VIP overflow, suggesting that NO causes prejunctional enhancement of VIP release in the stomach (Grider et al., 1992). Clearly, the relative roles of VIP-like peptides, NO (and ATP) in inhibitory NANC enteric transmission still need further clarification.

Another gas, carbon monoxide (CO), has also been suggested as a neuronal messenger involved in gastrointestinal motor control. The main source for endogenous CO is thought to be the degradation of heme to biliverdin by the enzyme heme oxygenase (HO) (Maines, 1988). The constitutive form of this enzyme, HO-2, has recently been demonstrated in local myenteric neurons (Ny et al., 1995b). CO relaxes intestinal smooth muscle and elevates cGMP. Zinc-protoporphyrin-IX (Zn PP), which inhibits CO formation by HO, has been reported to inhibit electrical field stimulation (EFS)-evoked relaxations of the opossum internal anal sphincter (Rattan and Chakder, 1993). Also, the VIP-evoked relaxation in this preparation was inhibited by Zn PP. However, Ny et al. (1995b) did not find any effect by Zn PP on the NANC relaxation in the cat esophageal sphincter. Furthermore, the relaxation by VIP of rat aorta was attenuated by Zn PP, probably via a plasma membrane effect, rather than by reduced CO production (Ny et al., 1995a). The messenger role(s) for CO in autonomic transmission clearly remains to be more thoroughly studied in the future.

IV. Neuropeptide Y/Adenosine 5′-Triphosphate In Sympathetic Nerves

A. Neuropeptide Y as Neurotransmitter

1. Biosynthesis, release and degradation. Neuropeptide Y is a 36-amino-acid peptide and the neuronal counterpart to the peptide YY (peptide with N-terminal and C-terminal tyrosine, Y being the abbreviation for tyrosine in the single letter amino acid code) which is mainly present in intestinal endocrine cells (Lundberg et al., 1982c). Neuropeptide Y was isolated from porcine brain and sequenced in 1982 (Tatemoto, 1982). The demonstration of NPY in a subpopulation of sympathetic neurons also containing NA (Lundberg et al., 1982d, 1983e) (fig. 8) was predicted from earlier data using

antisera to a related peptide, i.e., avian pancreatic polypeptide. NPY forms a tertiary structure consisting of an N-terminal polyproline helix (residues 1 to 8) and an amphiphilic α-helix (residues 15 to 30) connected with a β-turn creating a hairpin-like loop, which is sometimes referred to as the PP fold (Schwartz et al., 1990). The amidated C-terminal end (residues 30 to 36) projects away from the hairpin loop (Allen et al., 1987; MacKerrel et al., 1989). The gene encoding NPY was initially obtained from pheochromocytoma tumors (Minth et al., 1984, 1986). In the prohormone, mature NPY(1–36) is flanked at its C-terminus by 33 amino acids, three of which are the Gly-Lys-Arg motif necessary for NPY amidation. This amidation is critical for the biological effects of NPY with the exception of mast cell degranulation, (Shen et al., 1991). The peptide formed by the remaining 30 amino acids of the precursor has been named C-terminal flanking peptide of NPY. This peptide is coreleased with NPY, and, although no function has yet been assigned to this peptide (Allen et al., 1987), large peptide precursor molecules may hide interesting biological activities of specific cleavage products. NPY gene expression is stimulated by phorbolesters and nerve growth factor (Sabol and Higuchi, 1990).

Nerve activity seems to be an important regulator of NPY synthesis because the increased firing rate of sympathetic neurons seen after reserpine treatment (Pernow et al., 1988d) is associated with elevated levels of NPY mRNA and axonal transport of the peptide (Schalling et al., 1991). Conversely, after blockade of ganglionic nicotine N2-receptors or surgical preganglionic denervation, the expression of NPY mRNA in sympathetic ganglion cells decreases (Schalling et al., 1991).

After synthesis, NPY is stored in LDV and axonally transported to terminal regions (Fried et al., 1985a, b) (table 4, fig. 8). Vinblastine, an antitumor agent that impairs microtubuli function and thereby axonal transport, causes reduction of NPY levels in terminal regions, platelets and increase of the peptide in ganglia of rats (Hemsén et al., 1991). Release of NPY can be detected locally as overflow of NPY-like immunoreactivity (LI) from various vascular beds and can be evoked in experimental animals both by electrical nerve stimulation (Lundberg et al., 1984a; Rudehill et al., 1986; see Lundberg et al., 1990) and by reflexogenous sympathetic activation, for example, hemorrhagic hypovolemia or endotoxin shock and during physical exercise in humans (Lundberg et al., 1985b; Rudehill et al., 1987; Pernow et al., 1986b, 1988c, 1990). However, release of enough NPY to increase systemic plasma NPY levels in humans requires strong sympathetic activation (Lundberg et al., 1985b; Pernow et al., 1986b). Plasma NPY levels are likely to represent spillover after local release and in analogy to plasma NA, plasma NPY can be used as an indicator of high sympathetic tone rather than documenting NPY concentrations at its site of receptor activation. In experimental animals, it was demonstrated

TABLE 4
Transmitter characteristics (NPY, ATP, NA)

	NPY		ATP		NA	
Synthesis	cell body precursor		oxidative phosphorylation		enzymes (TH,DBH)	
Storage	LDV		SDV		SDV	
			(LDV)		LDV	
Terminal resupply	axonal transport		local synthesis		local synthesis reuptake	
Preferred receptors	Y_1	Y_2	Y_3	P_{2X1}	P_{2y}	α_1	α_2
Selective agonist	($Leu^{31}Pro^{34}$) NPY	N-acetyl Leu^{28} Leu^{31} NPY(24–36)	NPY>> PYY	α,βmetATP	2-SATP	phenylephrine	UK 14304
Antagonist	BIBP 3226 SR120819A SR120107A	–	–	$ANAPP_3$ α,βmetATP suramin	suramin RB2 CB3GA	prazosin	yohimbine
Second-messenger	Ca^{2+} cAMP	Ca^{2+} cAMP	Ca^{2+} cAMP	Ca^{2+}	IP_3, Ca^{2+} PKC, NO	IP_3, Ca^{2+}	K^+,Ca^{2+} cAMP
Functional response	vasoconstriction	inhibition of transmitter secretion (vasoconstriction)	inhibition of chromaffin cell secretion rat colon contraction	smooth muscle contraction	inhibition of transmitter secretion vasodilation	vasoconstriction	inhibition of transmitter secretion (vasoconstriction)
Removal	peptidase (NEP)			ectoATPase	reuptake		

Summary of transmitter characteristics for NPY, ATP and NA, which coexist in sympathetic postganglionic neurons and act as vasoconstrictors.
TH, tyrosine hydroxylase; DBH, dopamine-β-hydroxylase; SDV, small dense-cored vesicles; LDV, large dense-cored vesicles; α,βmetATP, α,βmethyleneATP; 2-SATP, 2-methylthio-ATP; $ANAPP_3$, arylazidoaminopropriny ATP; RB2, reactive blue 2; CB3GA, cibacron blue 3GA; IP_3, inositoltrisphosphate; PKC, protein kinase C.
(For further subdivision and characteristics of adrenergic receptors, see Bylund et al., 1994; for comments about problems of classifying P_2 receptors, see Kennedy and Leff, 1995.)

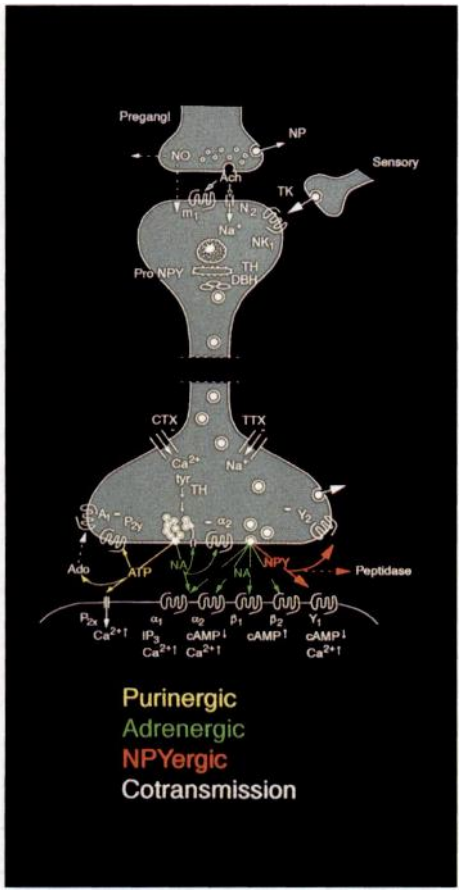

FIG. 8. Schematic illustration of a sympathetic neuron containing multiple transmitters: NA, ATP and NPY. A preganglionic cholinergic terminal and an axon collateral from a capsaicin-sensitive sensory neuron have established contacts with this multitransmitter postganglionic neuron. Local synthesis of NA occurs mainly in nerve endings, from the amino acid Tyr with a series of enzymes (e.g., tyrosine hydoxylase, TH, and dopamine-β-hydroxylase, DBH). NA is stored in both small and large dense-cored vesicles via a reserpine-sensitive uptake. After release, NA activates a variety of postjunctional α-adrenoceptors and β-adrenoceptors linked to IP_3 or cAMP formation, and prejunctional α_2-adrenoceptors, which inhibit Ca^{2+} fluxes, thus reducing further release. The bulk of the released NA is then taken up into the nerve terminal by a desipramine-sensitive pump and reused. ATP is also stored in vesicles and is, like NA, released as quanta, even upon single impulses (which for ATP can be detected by electrophysiological changes, EJP, in, for example, smooth muscle cells). ATP acts on rapid ionotropic P_{2x1} receptors, leading to Ca^{2+} influx, depolarization, and contraction of smooth muscle (in blood vessels and vas deferens). Furthermore, ATP may activate prejunctional P_{2y} receptors that inhibit transmitter release. Released ATP is degraded by ecto ATPase to products such as adenosine (Ado), which may also act prejunctionally on A1 receptors inhibiting transmitter release. NPY is synthesized in the cell body as precursor, stored in large dense-cored vesicles and axonally transported to terminal regions. After release, NPY may either activate postjunctional receptors (mainly Y_1 type, except in the spleen, where Y_2 type also evokes vasoconstriction), leading to contraction of vascular smooth muscle (via Ca^{2+} mobilization and cAMP inhibition), or prejunctional Y_2 receptors, inhibiting transmitter release. NPY is cleared from release sites by diffusion and enzymatic degradation. Release of all three transmitters involves TTX-sensitive depolarization and influx of Ca^{2+} through ω-conotoxin (CTX)-sensitive channels. It is clear that these three transmitters have distinct differences regarding time span of action. Thus, ATP acts in milliseconds, NA in seconds and NPY in minutes, depending both on receptor second-messenger characteristics and rate of transmitter removal from the sites of action. Furthermore, there is evidence that NPY is released mainly upon stronger activation due to its exclusive storage in LDV, and powerful α_2-inhibition by endogenous NA. A variety of classical sympatholytic drugs, such as reserpine, guanethidine and clonidine, exert complex actions on NA, ATP and NPY mechanisms (table 5). At the cell body level, cholinergic ganglionic transmission involves nicotinic (N2) receptors with some facilatory contribution by m1 receptors. The presence of NOS in preganglionic nerves suggests that NO can be formed from L-arginine (Arg) and possibly modulate ganglionic transmission. Furthermore, tachykinins such as SP released from sensory axon collaterals may also facilitate ganglionic transmission via NK_1 receptor-mediated slow EPSP. Preganglionic nerves in addition contain LDV with NPs such as endogenous opioids.

TABLE 5

Comparison of the effects of various classical sympathoactive drugs on release of the sympathetic transmitters NPY, ATP and NA[c]

	NPY release	ATP release	NA release
clonidine	−	−	−
yohimbine	+	+	+
desipramine	−	−	−[a]
tyramine	0	0	+
guanethidine	−	−	+, −
reserpine	+[b]	+	−

−, inhibition; +, stimulation; 0, no effect.

[a] After desipramine, NA overflow is increased while true nerve-evoked release is decreased.

[b] Initial release is followed by decreased release.

[c] The reserpine-evoked changes in NPY, ATP and NA release have different mechanisms: the effect on NA depends on inhibition of granular uptake and storage, while the enhanced NPY release, leading to tissue depletion, is due to increased nerve impulse traffic in combination with loss of prejunctional α_2-adrenergic inhibition in the absence of NA. ATP release is enhanced, whereas this mediator is not depleted by reserpine due to a lack of α_2 feedback.

that upon stimulation at higher frequencies, the increase in NPY-LI overflow was proportionally larger than that of NA (Lundberg et al., 1986, 1989; Pernow et al., 1989). Thus, the relative amounts of NPY and NA released in the neuroeffector junction may serve as a chemical code of stimulation frequency. Differences in release has been attributed to the partially different vesicular storage of NA and NPY (the latter agent being present only in LDV) (Fried et al., 1985a, b; see Lundberg et al., 1990). As discussed below, NPY release is prejunctionally regulated by endogenous NA acting on α_2-adrenoceptors: α_2-agonists such as clonidine inhibit and α_2-antagonists such as phentolamine increase stimulation-evoked NPY release. Consequently, after NA depletion by reserpine, NPY release is markedly enhanced. This prejunctional α_2-adrenoceptor regulation of NPY may explain why NPY release is suppressed upon low frequency stimulation and why it is difficult to deplete the tissue of NPY and thus eliminate NPY release under control conditions despite prolonged stimulation (Modin et al., 1994a). Prejunctional NPY (Y) receptor stimulation with PYY, has also been shown to suppress overflow

of NPY (Pernow and Lundberg, 1989a). Enhancement of NPY-LI release has been demonstrated upon stimulation of angiotensin II receptors (Pernow and Lundberg, 1989a) and β-adrenoceptors (Dahllöf et al., 1991), although these effects are quantitatively much less prominent than the inhibitory α_2-regulation.

More detailed studies comparing the mechanisms involved in release of NPY and NA have revealed that nerve stimulation-evoked release of both agents depends on influx of extracellular Ca^{2+} through CTX sensitive N-type channels (table 5) (Franco-Cereceda et al., 1989a; Haass et al., 1989a, b, 1990). Ischemia leads to a non-exocytotic release of NA that is similar to the effect of tyramine. In contrast, no outflow of NPY occurs upon moderate tissue ischemia (Franco-Cereceda et al., 1989a; Haass et al., 1989a,b). In accord, tyramine selectively releases NA but not NPY (Haass et al., 1989a; Lundberg et al., 1989d). In in vivo experiments, hypoxic conditions seem to enhance stress-evoked release of NPY compared with NA from the human heart (Kaijser et al., 1990, 1994).

Nearly a decade ago, it was demonstrated that circulating NPY disappears relatively slowly from plasma, with a half-life of approximately 5 minutes in both pig (Rudehill et al., 1987) and humans (Pernow et al., 1987b). The splanchnic circulation seems to have the highest capacity to remove NPY from plasma as revealed by local arteriovenous gradients in humans (Ahlborg et al., 1992). The enzymes involved in degradation of NPY and the related peptide PYY have recently been characterized. Thus, there is evidence that a somewhat shorter form of PYY, PYY 3–36, is formed in the tissue and that it also circulates in plasma (Eberlein et al., 1989). The corresponding cleavage in NPY, which shares the same N-terminal amino acids, is catalyzed by dipeptidyl peptidase IV (Mentlein et al., 1993). Interestingly, NPY(3–36) is likely to be a selective Y_2 receptor agonist. This suggests that a family of NPY peptides with different pharmacological profiles may be present, and N-terminal processing can produce ligands of different receptor sensitivity. After cleavage of NPY to NPY(3–36), this peptide may be further degraded by NEP to biologically inactive forms (Mediros and Turner, 1994).

In rats, circulating NPY appears to emanate primarily from platelets. Megakaryocytes from rats and certain autoimmune mice strains contain NPYmRNA (Eriksson et al., 1987, 1991). In contrast, no NPYmRNA was detected in normal human and pig bone marrow (Eriksson et al., 1991), which parallels the low circulating NPY plasma levels present in these species. Release of NPY as revealed by changes in plasma levels upon sympathetic activation is therefore much more difficult to study in rats compared with other species (see Lundberg et al., 1990), and specific precautions are necessary to avoid contamination of plasma with platelet-derived NPY.

2. Biological actions. Initial functional studies revealed that NPY, like PYY, caused long-lasting vasoconstriction mimicking the nonadrenergic response to sympathetic nerve stimulation (Lundberg and Tatemoto, 1982). Subsequently, NPY has been shown to reduce local blood flow in a variety of vascular beds (Rudehill et al., 1987) in different species, including humans. Thus, NPY-evoked vasoconstriction has been shown in skeletal muscle (Pernow et al., 1987b), heart (Clarke et al., 1987; Maturi et al., 1989), splanchnic circulation, kidney (Ahlborg et al., 1992; Pernow et al., 1987a) and brain (Edvinsson et al., 1984). In vitro, although most isolated large blood vessels from experimental animals do not respond to NPY with contractions (with rare exceptions such as guinea pig caval vein), NPY can serve as a regulating factor, enhancing the action of other vasoconstrictor agents (Ekblad et al., 1984; Pernow et al., 1986a). Because in the rat tail artery an apparent direct vasoconstrictor effect of NPY occurs only in the presence of the NA reuptake blocker cocaine and this contraction can be fully blocked by the α-receptor antagonist prazosin, spontaneously released NA may be involved and accordingly circulating, or locally released NA may be important in vivo to trigger NPY contractions (Glenn et al., 1995). However, NPY does seem to evoke contraction without NA reuptake blockade in small human vessels, including cerebral (Mejia et al., 1988), coronary (Franco-Cereceda and Lundberg, 1987), renal (Pernow et al., 1987a), skeletal muscle (Pernow and Lundberg, 1986) and subcutaneous arteries (Erlinge et al., 1993). The NPY contraction is independent of the endothelium (Pernow, 1989). Another prominent action of NPY is prejunctional inhibition of the release of NA, NPY itself and ATP (Lundberg et al., 1982d; Lundberg and Stjärne, 1984; Stjärne et al., 1986; Pernow et al., 1986a; Pernow and Lundberg, 1989a). A widely used bioassay organ for this NPY prejunctional effect is the vas deferens of mouse, rat and guinea pig (Lundberg et al., 1982d, 1984e; Stjärne et al., 1986). Furthermore, NPY inhibits transmitter release from parasympathetic nerves in the heart (Lundberg et al., 1984e; Potter, 1985, 1987), lower airways (Grundemar et al., 1988; Matran et al., 1989b), and from airway (Matran et al., 1989b; Grundemar et al., 1990b) and cardiac (Giuliani et al., 1989) sensory nerves. Parallel biochemical and electrophysiological studies have revealed that NPY inhibits SP release and Ca^{2+} currents in sensory neurons (Walker et al., 1988). Finally, it should be emphasized that NPY exerts long-term trophic actions because it stimulates proliferation of vascular smooth muscle cells (Shigeri and Fujimoto, 1993; Zukowska-Grojec et al., 1993; Erlinge et al., 1994).

3. NPY-receptors and antagonists. The initial subdivision of NPY receptors (named Y receptors) was based on pharmacological evidence gleaned using bioassay systems. Thus, the C-terminal fragment of NPY, NPY(13–36) had inhibitory prejunctional effects on transmitter-evoked contraction in the rat vas deferens (Y_2 receptor),

whereas it did not activate the Y_1 receptor that mediates constriction of vascular smooth muscle (Wahlestedt et al., 1986). However, in some vascular beds such as the pig spleen (Lundberg et al., 1988a; Modin et al., 1991) and the guinea pig heart (Rioux et al., 1986), NPY(13–36) was reported to evoke vasoconstriction, albeit with less potency than NPY(1–36). More selective agonists for the Y receptors have subsequently been developed confirming these findings; these include Leu^{31}, Pro^{34}NPY for Y_1 receptors (Fuhlendorff et al., 1990) and N-acetyl $Leu^{28,}$ Leu^{31}NPY(24–36) for Y_2 receptors (Potter et al., 1993; Lundberg and Modin, 1995) (table 4). A useful fact has been that certain neuroblastoma cell lines seem to express either Y_1 (SK-N-MC cells) or Y_2 (SMS-KAN cells) type receptors (Sheik et al., 1989; Rudolf et al., 1994), and these receptors may be glycoproteins of 70 kD and 50 kD, respectively (Sheik and Williams, 1990). Characteristic for the Y_1 receptor is the fact that, if NPY is truncated at the first N-terminal residue Tyr 1 to NPY(2–36), this results in a marked loss of biological activity or receptor affinity (Rioux et al., 1986; MacKerrel et al., 1989; Grundemar et al., 1992). Further truncations at the N-terminal yield fragments that are less potent still, or even inactive in Y_1 receptor assays. Binding to Y_2 receptors, on the other hand, does not seem to require an intact N-terminal end because NPY(2–36) or NPY(3–36) are still quite potent compared with NPY. An intact C-terminal end of NPY is required for activation of the Y_2 receptor, however. Thus, substitution in position 34 (as in Pro^{34}NPY) results in loss of affinity for the Y_2 receptor. ^{125}I(Leu^{31}, Pro^{34})PYY and ^{125}I(3–36)PYY can be used as highly selective radioligands for studies on Y_1 and Y_2 receptor subtypes, respectively (Dumont et al., 1995), in addition to the ^{3}H form of the nonpeptide Y_1 receptor antagonist BIBP 3226 (Entzeroth et al., 1995).

The Y_1 receptor was first found as an orphan receptor belonging to the G-protein-coupled family but with unknown ligand (Eva et al., 1990). Later, this sequence was recognized to be identical to the Y_1 receptor (Herzog et al., 1992; Larhammar et al., 1992). Acidic residues (aspartic acid 205 and 287) in extracellular loops of the Y_1 receptor are essential for binding, presumably forming salt bridges with arginine 33 and 35 of NPY (Walker et al., 1994). Cloning of the Y_2 receptor has also recently been reported (Rose et al., 1995). The human Y_2 receptor also belong to the G-protein-coupled receptors with seven putative transmembrane regions. The human Y_1 and Y_2 receptors have 31% identity at the amino acid level. The recombinant Y_2 receptor is functionally coupled to Ca^{2+} motilization and inhibition of forskolin-stimulated cAMP production (Rose et al., 1995). The Y_1 receptor has been shown to be coupled to both increases in intracellular Ca^{2+} and inhibition of stimulated adenylyl cyclase (Aakerlund et al., 1990; Herzog et al., 1992). In a Y_2 receptor dominated tissue, like pig spleen, NPY also inhibits stimulated cAMP formation (Lundberg et al., 1988a, b). Y_2 receptors on SMS-KAN cells interact directly with several inhibitory guanine nucleotide binding proteins (Freitag et al., 1995). The NPY-evoked increase in intracellular Ca^{2+} may be primarily due to release from intracellular stores, at least when regarding the direct contractile effect on human and pig vascular smooth muscle because it occurs in the absence of Ca^{2+} in the extracellular medium (Pernow and Lundberg, 1986). It has also been reported that inhibition of Ca^{2+} activated K^+ channels by NPY may contribute to its excitatory action on vascular smooth muscle cells (Xiong and Cheung, 1994). NPY inhibits Ca^{2+} influx into cultured rat dorsal root ganglion neurons via Y_2 receptors (Bleakman et al., 1991). It should also be mentioned that Y_1 receptor mRNA and protein is present in dorsal root sensory ganglion neurons of the small type (Zhang et al., 1994). NPY also inhibits Ca^{2+} currents in sympathetic neurons (Schofield and Ikeda, 1988). Presumably this occurs via interference with N-type Ca^{2+} channel (Wiley et al., 1990) by pertussis toxin-sensitive G-protein coupling (Walker et al., 1989). As with many prejunctional effects (Duckles and Budai, 1990), the ability of NPY to block neurotransmitter release is dependent on how strong stimulation is used. Thus, the inhibitory effect of NPY can be overcome by stimulating the presynaptic neuron more rapidly or with longer trains of action potentials. This ability of strong stimulation to overcome prejunctional inhibitory effects of NPY has its direct correlation in the ability of NPY to modulate Ca^{2+} entry into the neuron (Thayer and Miller, 1990). It seems likely that the ability of NPY to inhibit NA from sympathetic neurons is based on a similar mechanism on Ca^{2+} influx as for sensory neurons (Schofield and Ikeda, 1988). It is of interest that NPY is present only in a subpopulation of sympathetic neurons (see Lundberg et al., 1990) and Schofield and Ikeda (1988) reported that the inhibitory effect of NPY in bullfrog sympathetic ganglia correlated with the presence of NPY.

In the gastrointestinal tract, NPY is not only present in sympathetic nerves but also to a large extent in intrinsic neurons (see Furness et al., 1992b). NPY (and PYY) exerts potent antisecretory effects on epithelial ion and fluid transport (Saria and Beubler, 1985; Cox and Cuthbert, 1990). It has also been shown that NPY (at concentrations that do not alter blood pressure) causes increased absorption of water and ions under basal conditions. Furthermore, the antisecretory effect by NPY is more pronounced following secretagog pretreatment (Holzer-Petsche et al., 1991), suggesting that this peptide (like PYY) could inhibit diarrhea. Presumably, NPY exerts its antisecretory effect by Y_2 receptors (Cox and Cuthbert, 1990).

NPY has capacity to inhibit cholinergic and noncholinergic contractions by interference with enteric transmission (Holzer et al., 1987; Wiley and Owyang, 1987). In humans, gastric emptying is delayed by NPY and

PYY (Allen et al., 1984). NPY can inhibit Ca^{2+} currents in myenteric neurons (Hirning et al., 1990), which may explain these findings and suggests that they are Y_2 mediated.

Pharmacological evidence suggests the presence of an additional NPY receptor namned Y_3. At this receptor, PYY related peptides have characteristically no effect (table 4). The Y_3 receptor has been described in rat heart (Balasubramaniam et al., 1990), adrenal medulla (Higushi et al., 1988) and rat colon (Dumont et al., 1993). In the adrenal medulla, NPY but not PYY inhibits nicotine-stimulated catecholamine release and in rat colon in vitro, NPY but not PYY evokes contraction (Dumont et al., 1993). Also the Y_3 receptor mediated effects in bovine chromaffin cells may involve inhibition of the adenylate cyclase system (Nörenberg et al., 1995). Finally, pancreatic polypeptide, which also resembles NPY in structure, suppresses electrically evoked contractions in rat vas deferens with receptors distinct from Y_1 and Y_2 (Jörensen et al., 1990).

In addition to specific receptor mediated actions in high doses, NPY activates mast cells evoking release of e.g., histamine (Grundemar and Håkanson, 1991) which is likely to account for the hypotensive component of the biphasic blood pressure response in the rat (Grundemar et al., 1990a). NPY is also able to induce a flare response after intradermal injections in humans (Mousli et al., 1995). This property of NPY is even more pronounced for fragment 18–36 (Mousli et al., 1995) and is likely to be related to the ratio of the number of basic (such as arginine) to acidic amino acid residues (Johnson and Erdos, 1973) giving a net positive charge.

Recently, two potent series of selective Y_1 receptor antagonists have been developed (Rudolf et al., 1994; Serradeil-Le Gal et al., 1995). Thus, BIBP 3226, SR120819A and SR120107A are low molecular weight nonpeptide Y_1 receptor antagonists with nM affinity in several species including rat and humans. The corresponding S-enantiomer of BIBP 3226, BIBP 3435, is active on Y_1 receptors only in the μM range (Rudolf et al., 1994; Lundberg and Modin, 1995). In accord with the presence of Y_1 receptors in pig kidney and vascular Y_2 receptors in pig spleen, BIBP 3226 and SR120107A markedly inhibited the NPY and PYY-evoked vasoconstriction in the kidney but not in the spleen (Lundberg and Modin, 1995; Malmström et al., 1996). Furthermore, the hypertensive effect of NPY and PYY was markedly attenuated by SR120107A and BIBP 3226 but not by BIBP 3435 in agreement with the view that the main vascular NPY receptor is of Y_1 type. Both BIBP 3226 and 3435 somewhat lower blood pressure to a similar extent in higher doses (Lundberg and Modin, 1995) suggesting additional actions of these first generation of Y_1 receptor antagonists which possibly can be related to positively charged arginine-like moieties. Even after oral administration, SR120819A counteracts the pressor response of the Y_1 agonist (Leu^{31}, Pro^{34})NPY given i.v. in guinea pigs (Serradeil-Le Gal et al., 1995).

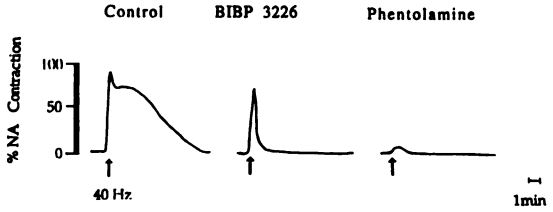

FIG. 9. Inhibitory effects of the Y_1 receptor antagonist BIBP 3226 (10^{-6} M) and the α-adrenoceptor blocking agent phentolamine, (10^{-6} M) on the electrical field stimulation contractile response (40 Hz for 10 seconds) in vitro in the presence of propranolol (10^{-6} M) in guinea pig thoracic caval vein. Note that BIBP 3226 inhibits the long-lasting component of the contraction, whereas phentolamine blocks the initial component, indicating NA and NPY as rapid and slow transmitters, respectively. Time scale indicates 1 minute.

4. NPY-ergic transmission. a. NANC VASOCONSTRICTION. Until recently, the only available antagonists of NPY effects, including benextramine or PP56, were either not selective or not sufficiently potent (Doughty et al., 1990; Pernow et al., 1992). After the introduction of BIBP 3226 and SR120107A it has now been possible to finally establish that endogenous NPY acting via Y_1 receptor mechanisms is likely to play a role in evoking the long-lasting sympathetic vasoconstriction seen in guinea pig caval vein (fig. 9) (Malmström and Lundberg, 1995a, b). Furthermore, some reduction of the duration of the vasoconstrictor response to sympathetic nerve stimulation by BIBP 3226 but not by the inactive enantiomer BIBP 3435 can also be observed in vivo in pig hind limb (mainly reflecting effects on skeletal muscle), nasal mucosa and skin. The single impulse response, as well as the peak vasoconstriction, to high frequency stimulation were not influenced by BIBP 3226, however (Lundberg and Modin, 1995). These findings are consistent with the view that NPY is a cotransmitter with NA (and ATP), especially upon strong activation (see Lundberg et al., 1990).

The involvement of NPY in nonadrenergic sympathetic vascular control may also be studied using BIBP 3226 combined with α- and β-adrenoceptor blockade or NA depletion by reserpine treatment. As discussed below, the reserpine treatment should be combined with interruption of nerve activity in order to maintain tissue levels of NPY in terminal regions of cardiovascular sympathetic nerves (Lundberg et al., 1990).

In the cat submandibular gland (Lundberg and Tatemoto, 1982), it was initially demonstrated that NPY mimicked the prolonged vasoconstriction evoked by high frequency sympathetic nerve stimulation in the presence of α- and β-adrenoceptor antagonists. This long-lasting effect grows progressively smaller upon repeated activation, in contrast to the rapid reproducible purinergic sympathetic vasoconstriction described below. Interestingly, guanethidine blocks both the nonadrenergic sympathetic vasoconstriction and NPY release from the spleen (Lundberg et al., 1984a). Such long-lasting vaso-

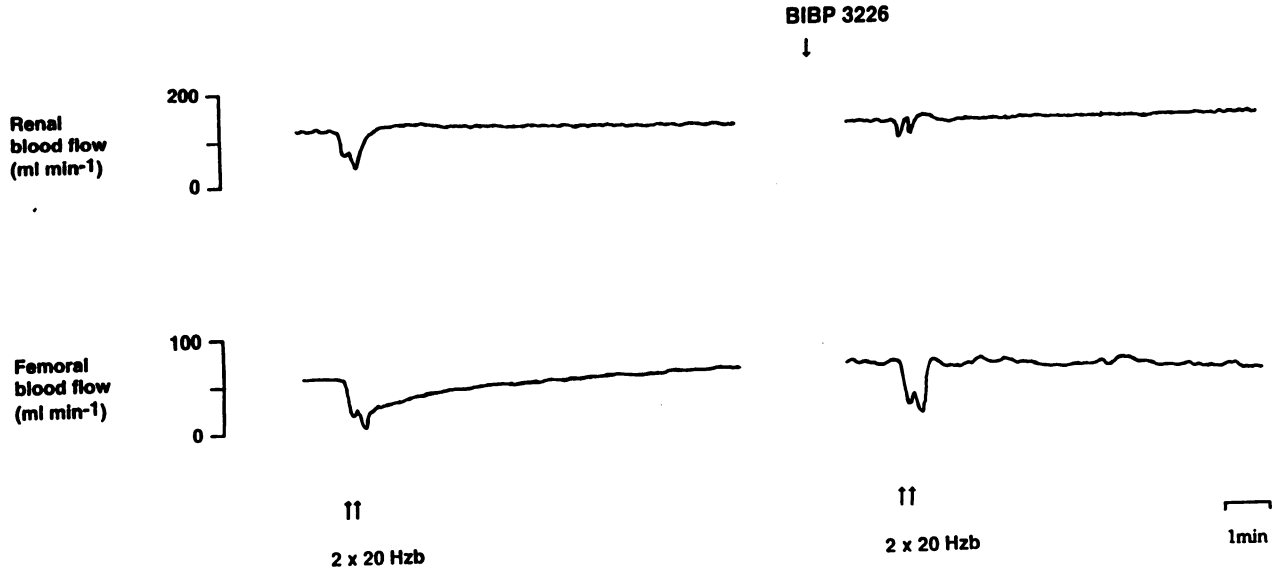

FIG. 10. Effects of the Y_1 receptor antagonist BIBP 3226 on the vasoconstrictor response in vivo to sympathetic nerve stimulation at high frequency (two 1-second bursts at 20 Hz with 10-second intervals, 2 × 20 Hzb), in renal artery (*upper panel*) or femoral artery (*lower panel*) in reserpinized pigs lacking NA. Note that BIBP 3226 inhibits the maximal effect in the kidney, but only the long-lasting component in femoral artery. Bar represents 1 minute. This suggests that NPY-ergic transmission is dominating in the kidney after reserpine, whereas mainly the long duration of the vasoconstrictor response in femoral artery can be attributed by NPY (or rather Y_1 receptor activation). The remaining rapid short-lasting nonadrenergic component is likely to depend on purinergic transmission.

constriction, resistant to adrenoceptor blocking agents, has been demonstrated in many other vascular beds in vivo including skeletal muscle (Pernow et al., 1988a; Modin et al., 1993), kidney (Pernow and Lundberg 1989b), and nasal mucosa (Lundblad et al., 1987b; Lacroix et al., 1988). Furthermore, vasoconstriction remaining after reserpine treatment, when the tissue content of NA is reduced by >90%, is also present in these vascular beds (Lundberg et al., 1986; Lundblad et al., 1987b; Lacroix et al., 1988; Pernow et al., 1988b; Pernow and Lundberg, 1989b). The involvement of NPY as a mediator of sympathetic nonadrenergic vasoconstriction was supported by several additional observations: (1) tachyphylaxis to NPY or Y_1 receptor agonists inhibits nonadrenergic sympathetic nerve responses (Öhlén et al., 1990; Morris, 1991; Morris and Sabesan, 1994); (2) NPY overflow is highly correlated to the nonadrenergic vasoconstrictor responses, especially the decline upon repeated stimulation (Lundberg et al., 1989d; Modin et al., 1993); (3) NPY levels in plasma escaping into the pig splenic venous effluent upon stimulation are clearly within the vasoconstrictor range (nM) (Rudehill et al., 1987; Lundberg et al., 1989b; Modin et al., 1995); (4) the development of supersensitivity to NPY-evoked vasoconstriction in the pig nasal mucosa (Lacroix and Lundberg, 1989) or rat tail artery (Neild, 1987) after sympathetic denervation also favors a role for endogenous NPY in regulation of the sensitivity of vascular Y receptors.

Experiments using BIBP 3226 and SR120107A have revealed that Y_1 receptor mechanisms are of crucial importance for the long-lasting component of the reserpine resistant sympathetic vasoconstriction in response to high frequency stimulation in pig spleen, kidney, nasal mucosa and hind limb (fig. 10). Furthermore, the maximal amplitude of the vasoconstriction was reduced, mainly in the kidney (where Y_1 receptors predominate) (fig. 10) but also to some extent in the spleen (Lundberg and Modin, 1995; Malmström et al., 1996). In contrast, the vasoconstriction response to single impulse stimulation (which was only observed only in nasal mucosa and hind limb) in the presence of BIBP 3226 and SR120107A was largely uninfluenced in reserpinized pigs. It was also established that BIBP 3226 did not influence NA release in controls or NPY release in reserpinized pigs (Lundberg and Modin, 1995). This shows that endogenous NPY, acting on Y_1 receptors plays an important role in the long duration of NANC vasoconstriction to high frequency stimulation, whereas other mediators, such as ATP, mediate the single impulse responses and the peak amplitude of the effects in skeletal muscle and nasal mucosa. In the kidney, the NANC vasoconstriction seems to be largely Y_1 receptor-mediated although this response is of short duration (fig. 10) presumably due to powerful autoregulatory mechanisms in this organ. Interestingly, endogenous NPY released from sympathetic nerves in the spleen mainly activate Y_1 receptors considering the inhibitory effects of BIBP 3226 and SR 120107A, while circulating NPY (and PYY) preferentially influence Y_2 receptors which are resistant to these antagonists (Lundberg and Modin, 1995; Malmström et al., 1996).

In addition to direct vasoconstrictor actions, NPY enhances the vasoconstrictor effects of a variety of agents including NA (Ekblad et al., 1984; Pernow et al., 1986a).

The relevance of this remains to be established in vivo, however, because the potentiation is most pronounced on larger vessels where NPY *per se* exerts minor or no contractile effect. BIBP 3226 had only marginal effects on the maximal vasoconstrictor response to sympathetic nerve stimulation in controls, and therefore this facilitation by Y_1 receptors of NA and ATP mechanisms may not be of obvious importance in regulation of tissue blood flow. It is also of interest that NPY via Y_1 receptors stimulates DNA synthesis in vascular smooth muscle cells (Shigeri and Fujimoto, 1993; Erlinge et al., 1994) and NPY stimulate hypertrophy of adult ventricular cardiomyocytes (Millar et al., 1994). This implies that long-term treatment with an NPY receptor antagonist may influence medial thickness of blood vessels as well as cardiac hypertrophy.

b. PREJUNCTIONAL INHIBITION OF TRANSMITTER RELEASE. It has long been known that NPY inhibits sympathetic nerve-evoked responses in rat vas deferens (Lundberg et al., 1982d). The inhibition of NA release from this tissue parallelled the inhibition of contractions (Lundberg and Stjärne, 1984), and reduction of rapid stimulus-evoked but not spontaneously occurring excitatory junction potentials (EJP) in smooth muscle cells caused by ATP (Stjärne et al., 1986). This suggested prejunctional inhibitory mechanisms by NPY acting on transmitter release (both ATP and NA) from sympathetic nerve endings. Supportive data were also obtained from in vitro studies monitoring inhibition of NA release from vas deferens (Lundberg and Stjärne, 1984) and perivascular nerves (Pernow et al., 1986a; Lundberg et al., 1989b). Later studies showed that Y receptor stimulation (in this case with PYY) also reduced the release of NPY from sympathetic nerves in vivo (Pernow and Lundberg, 1989a). This prejunctional inhibitory effect of NPY on transmitter release from sympathetic nerves occurs independently of α_2-adrenoceptors (Lundberg and Stjärne, 1984; Stjärne et al., 1986) (fig. 8). Because NPY potentiated the contractile effects of α,β methylene ATP and NA in mouse vas deferens, NPY may have a dual role in this organ, initially locally to potentiate the contractile response to NA and ATP and subsequently to "turn off" secretion of transmitter (Stjärne et al., 1986). The prejunctional inhibitory effect of NPY on secretion of sympathetic transmitters is usually Y_2 mediated as revealed by in vitro experiments mainly on rat vas deferens (Wahlestedt et al., 1986; Grundemar and Håkanson, 1990; Modin et al., 1991). In the rabbit vas deferens, a Y_1 receptor agonist can inhibit responses caused by sympathetic transmitter release, however, suggesting the presence of prejunctional Y_1 receptors in this species (Doods and Krause, 1991). SR120819A exerted potent competitive antagonistic actions on the (Leu^{31}, Pro^{34})NPY inhibition of rabbit vas deferens contractions, confirming the presence of Y_1 receptors (Serradeil-Le Gal et al., 1995). Because the selective Y_1 antagonists BIBP 3226 and SR120107A did not influence the prejunctional effect of NPY in rat vas deferens (Rudolf et al., 1994) or change nerve stimulation-evoked NA or NPY overflow from pig kidney and spleen (Lundberg and Modin, 1995; Malmström et al., 1996), it is likely that Y_1 receptor mechanisms are not of major importance for regulation of transmitter secretion in these latter tissues.

NPY also exerts inhibitory actions on ACh release from vagal cholinergic nerves in the heart (Lundberg et al., 1984e; Potter, 1985, 1987, 1988). From a series of investigations in the dog in vivo, it has been suggested that NPY released from cardiac sympathetic nerves upon high frequency stimulation can cause a long-lasting depression of cardiac vagal tone (Hall et al., 1990; Warner and Levy, 1989; Warner et al., 1991). A similar inhibitory response on vagal function to sympathetic stimulation remains after adrenoceptor blockade (Potter, 1988; Warner and Levy, 1989) and reserpine treatment (Moriarty et al., 1993) but disappears after guanethidine (Potter, 1988), which together with the long duration of the effect is highly suggestive of an NPY-mediated response. The cardiac antivagal action of NPY is probably Y_2 receptor-mediated (Potter et al., 1989). Recent indirect evidence also suggests that sympathetic nerve stimulation attenuates parasympathetic vasodilation via NPY release acting on prejunctional Y_2 receptors (Lacroix et al., 1994) (fig. 6).

5. NPY and pathophysiology. At an early prehypertensive state, spontaneously hypertensive rats have a more dense perivascular innervation with NPY-containing fibers compared with control rats (Dhital et al., 1988; Lee et al., 1988). Furthermore, spontaneously hypertensive rats have greater pressor responsiveness to NPY in parallel with development of hypertension (Miller and Tessel, 1991). The relevance of NPY in this model remains to be tested using Y_1 antagonists. In addition, there is evidence that the prejunctional effect of NPY on NA release is less strong in spontaneously hypertensive rats (Westfall et al., 1987), a finding that is shared with other mediators in states of chronic hypertension (Tsuda and Masuyama, 1991). Circulating NPY levels are elevated, compared with normals, in patients with cardiovascular disease, such as acute myocardial infarction and angina pectoris (Ullman et al., 1990), heart failure (Hulting et al., 1990), and hypertension (Chalmers et al., 1989; Solt et al., 1990; Lettgen et al., 1994), where sympathetic nerve activity is increased (Anderson et al., 1989). Depletion of both NA and NPY from human heart tissue has been reported in patients suffering from severe and long-lasting heart failure associated with prolonged sympathetic activation (Anderson et al., 1992). Furthermore, the exercise-evoked increase in plasma NPY seems to be blunted in patients with moderate heart failure (Ullman et al., 1994b), indicating that synthesis is not sufficient to replace NPY during conditions of prolonged increase of nerve activity (see Lundberg et al., 1989c). Plasma NPY seems to have a prognostic value

for survival, especially in patients with cardiac failure (Ullman et al., 1994a).

NPY is a potent vasoconstrictor in humans in vivo, for instance, in the forearm circulation (Pernow et al., 1987b) with even lower threshold effects, approximately 200 to 300 pM, in the renal vascular bed and splanchnic region (Ahlborg et al., 1992a, b). Circulating plasma levels of NPY in patients with severe cardiac failure (Hulting et al., 1990) are clearly within this range. Intracoronary injection of NPY in patients with angina pectoris causes symptoms and EKG changes typical for cardiac ischemia (Clarke et al., 1987) in accordance with in vitro data that NPY contracts small human coronary vessels (Franco-Cereceda and Lundberg, 1987). The recent development of Y_1 receptor antagonists could therefore be of interest to prevent stress-evoked myocardial ischemia and hypertension. Furthermore, because NPY inhibits vagal activity, a Y_2 antagonist may be of relevance in the treatment of stress-evoked cardiac arrhythmias. One potential advantage with an NPY antagonist compared with drugs influencing other transmitters involved in sympathetic vasoconstriction is that basal vascular tone or mild reflex adjustments are less likely to be influenced (in contrast to α-adreno- or P_{2x}-receptor blocking agents).

Finally, because topical NPY acts as a nasal vasoconstrictor, a stable Y_1 agonist may represent a novel nasal decongestant (Baraniuk et al., 1992). Because NPY can inhibit gastrointestinal motility (Holzer et al., 1987) and exert antisecretory actions (Holzer-Petsche et al., 1991), a Y_2 agonist may represent a useful antidiarrheal agent (Cox and Cuthbert, 1990).

B. Adenosine 5′-Triphosphate as Neurotransmitter

1. Synthesis, release and degradation. Adenosine 5′-triphosphate is costored and coreleased with catecholamines from adrenal chromaffin cell granules (Carlsson et al., 1975). Furthermore, ATP is contained together with NA in sympathetic nerve terminal vesicles (Lagercrantz and Stjärne, 1974; Fried, 1980). Burnstock (1976) suggested that ATP was released together with NA from adrenergic nerves, although it was difficult to distinguish experimentally between "transmitter ATP" exocytotically released from sympathetic nerves and "nontransmitter ATP" originating from a variety of excitable cells (Stjärne, 1989). The increase in ATP overflow as determined by biochemical methods was Ca^{2+}-dependent, TTX-sensitive and blocked by guanethidine (Lew and White, 1987). However, in some tissues such as rabbit aorta (Sedaa et al., 1990), most (> 80%) of the ATP released by field stimulation was classified as originating from nonneuronal sources. More recently, using electrophysiological analysis of EJPs in smooth muscle cells, the release of neuronal ATP can be analyzed with much better tissue resolution and accuracy.

According to the vesicle hypothesis (Del Castillo and Katz, 1954, 1957), transmitters are released in multimolecular packets (quanta) equal to the individual vesicle content. The spontaneously occurring EJP in vascular or vas deferens smooth muscle cells probably represents release of single quanta of ATP (Westfall et al., 1978; Blakely and Cunnane, 1979; Cunnane and Stjärne, 1982, 1984; Burnstock, 1986a, b; Brock and Cunnane, 1988) (table 5). The nerve impulse-evoked release of ATP is also quantal (Blakeley and Cunnane, 1979) but intermittent, due to the depolarization-secretion coupling in the varicosities (Brock and Cunnane, 1988; Åstrand and Stjärne, 1989). During trains of high impulse frequency stimulation, some varicosities can probably release several quanta into the same junctional cleft. The per pulse quantal release of ATP declines progressively and profoundly during high frequency stimulation (Stjärne et al., 1994). Presumably, NA and ATP release are largely parallel. Thus, by using small carbon fiber electrodes and continuous amperometry in vitro (Gonon et al., 1993), release of NA was also demonstrated to occur intermittently when evoked by trains of electrical stimuli at 0.1 Hz, i.e., single pulses (Msghina et al., 1992; Msghina and Stjärne, 1993).

The released ATP is rapidly inactivated enzymatically by extracellular enzymes such as adenosine 5′-triphosphatase (ecto-ATPase) (Gordon, 1986; Cunnane and Manchanda, 1988). Presumably, the released ATP is inactivated within 100 milliseconds (Bao, 1993) (table 4, fig. 11).

2. Biological actions. ATP can act both as a vasodilator via actions on endothelial cells and as a vasoconstrictor via direct actions on vascular smooth muscle. Furthermore, ATP is a powerful contractile agent of the vas deferens and urinary bladder. Characteristic of the contractile response to ATP is that it is rapid in onset but short-lasting (Burnstock, 1986a, b, 1990). It should also be emphasized that ATP is rapidly broken down to adenosine which is also a powerful vasodilator, acting directly on vascular smooth muscle (Burnstock and Kennedy, 1986). Adenosine exerts powerful prejunctional inhibitory actions on transmitter release from sympathetic nerves (Fredholm et al., 1983), thereby reducing overflow of both NA and NPY (fig. 8) (Haass et

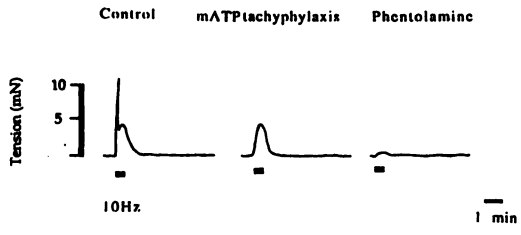

FIG. 11. Effects of α,β-methylene ATP tachyphylaxis and subsequent addition of the α-adrenoceptor blocking agent phentolamine on the biphasic response of guinea pig vas deferens to electrical field stimulation (10 Hz for 10 seconds, bar). Time scale indicates 1 minute. Note that purinergic P_{2X1} transmission accounts for the initial very short-lasting rapid phase, whereas the second phase is mediated by NA acting on α adrenoceptors.

al., 1989a). In analogy, the adenosine antagonist theophylline can enhance release of NA and NPY upon hypoxia, a condition known to be associated with very high adenosine levels (Thoresen et al., 1992).

3. Adenosine 5′-triphosphate receptors and antagonists. Adenosine and ATP receptors have been designated P_1- and P_2- purinoceptors, respectively (Burnstock, 1978). The prejunctional P_1-receptors are of the adenosine A1 subtype (Fredholm et al., 1983) (fig. 8). Later, the P_2-category has been further subdivided into two major classes, P2x- and P2y-purinoceptors (Burnstock and Kennedy, 1985). The excitatory effects of ATP in vascular smooth muscle are exerted via P2x-purinoceptors, which represent agonist gated ion channels, of which the receptor is an integral part (Benham, 1992a, b). This receptor protein P_{2X1} has recently been cloned in, for example, vas deferens (Valera et al., 1994). The overall structure of this receptor resembles epithelial Na^+ channels and mechanosensitive channels (Suprenant et al., 1995). The P_{2X1}-receptor can be blocked by arylazidoaminopropionyl ATP, α,β-methylene ATP tachyphylaxis (fig. 11), or by suramine (developed many years ago for treating trypanosome infections) (Burnstock, 1990). Activation of P_{2X1}-purinoceptors increases cation conductances, leading to EJP which may or may not fire a smooth muscle action potential. The membrane depolarization activates voltage-gated Ca^{2+} channels, which increases the influx of extracellular Ca^{2+} and triggers contraction (fig. 8) (Benham, 1992a,b).

Presynaptic P_{2y}-purinoceptors are G-protein coupled and inhibit NA release from sympathetic nerves, possibly as autoreceptors activated by endogenous ATP (see von Kügelgen et al., 1995) (fig. 8). Recently, suramine has also been reported to influence P_{2y}-receptors present on sympathetic nerves or linked to e.g., NO production from endothelial cells, and this agent can also inhibit ecto-ATPase, thereby confounding the simple competitive antagonism of endogenously released ATP (Crack et al., 1994; Kennedy and Leff, 1995b). Other P_{2y}-receptor antagonists, such as reactive blue 2 or cibacron blue 3GA, inhibit NANC relaxations in guinea pig pulmonary arteries, a response that also involves NO formation (Liu et al., 1992a, b) or prejunctional P_{2y} effects on sympathetic nerves respectively (von Kügelgen et al., 1995).

4. Purinergic transmission. a. VAS DEFERENS. In the vas deferens of rat, mouse and guinea pig, the neurogenic mechanical response is biphasic, consisting of an initial short-lasting twitch followed by a slower, maintained contraction (fig. 11). The initial twitch phase is nonadrenergic (Ambache and Aboo-Zar, 1971; Swedin, 1971) and blocked by arylazidoaminopropionyl ATP (Fedan et al., 1981) as well as by α,β-methylene ATP tachyphylaxis (Meldrum and Burnstock, 1983) or suramine (Mallard et al., 1992). The purinergic twitch component is blocked by nifedipine and potentiated by BayK 8644 being antagonist and agonist of L-type Ca^{2+} channels, respectively, in agreement with the involvement of voltage-dependent L-type Ca^{2+} channels (Blakeley et al., 1981; Stjärne et al., 1986). Therefore, ATP should be considered as a fast excitatory ionotropic transmitter (fig. 8). At the prejunctional site, inhibition of P_{2y} autoreceptors by e.g., cibacron blue 3GA or suramine, enhances NA release (von Kügelgen et al., 1994).

In studies of the human vas deferens (Birmingham, 1968; Anton and McGrath, 1977; Hedlund et al., 1985), it was, however, concluded that the sympathetic nerve stimulation-evoked contraction was purely adrenergic (α-receptor mediated), and no initial twitch contraction was observed corresponding to that caused by ATP described above, emphasizing that there are clear species variations.

b. BLOOD VESSELS. In many isolated blood vessels, the mechanical and electrical (EJP) smooth muscle responses to perivascular sympathetic nerve stimulation seem to involve a purinergic component, although the relative importance of ATP varies between different vessels (Burnstock, 1990). In the rabbit jejunal arteries, ATP has been claimed as the sole mediator of the contractile response to sympathetic nerve stimulation, whereas NA acts as a prejunctional modulator (Ramme et al., 1987). As in the vas deferens, the L-channel blocker nifedipine selectively inhibits the purinergic component of the sympathetic vasoconstriction in the dog mesenteric artery (Omote et al., 1989) and pithed rat (Bulloch and McGrath, 1988b). Short bursts of electrical impulses lasting approximately 1 second appear to favor the ATP-mediated contractile component over that mediated by NA (Kennedy et al., 1986; Bulloch and Starke, 1989). A contribution of ATP to sympathetic vasopressor responses of the pithed rat in vivo has also been demonstrated (Bulloch and McGrath, 1988a; Schlicker et al., 1989), again being sensitive to nifedipine (Bulloch and McGrath, 1988b). An excitatory NANC purinergic component in the sympathetic control of the intestinal circulation of the cat (Taylor and Parsons, 1989) and the dog (Muramatsu et al., 1989) and pulmonary circulation in the rat (Inoue and Kannan, 1988) has also been suggested. It should be emphasized that α,β-methylene ATP desensitization in vivo has been reported to influence NPY overflow, suggesting impairment of release (Lacroix et al., 1989; Lundberg et al., 1989d).

Studies on the relative importance of NA and ATP as mediators of the contractile response of the rat tail artery to sympathetic stimulation suggest age-related variations: the purinergic contribution was larger in young animals (Bao et al., 1989). In humans, there is as yet no conclusive evidence for the role of purinergic transmission in vascular control, and Rump and von Kügelgen (1994) recently suggested that NPY rather than ATP was likely to mediate a nonadrenergic contractile component to nerve stimulation in the human saphenous vein. Thus, the P_{2x}-antagonist suramine

failed to influence the contractile response to nerve stimulation.

c. URINARY BLADDER AND INTESTINE. The NANC excitatory transmission in the urinary bladder (Ambache and Aboo-Zar, 1970) has also been demonstrated to blocked by α,β-methylene ATP tachyphylaxis (Kasakov and Burnstock, 1982). Because also botulinum toxin inhibits this component, cholinergic nerves may release ATP (MacKenzie et al., 1982). The importance of ATP excitation in control of human urinary bladder motility is less obvious, however (Andersson, 1993). ATP has also been considered as a major candidate to mediate the fast apamin-sensitive IJPs mediating relaxation in the intestine (Shaba and Vladimirova, 1980; Zagorodnyuk et al., 1995).

5. Adenosine 5′-triphosphate and pathophysiology. ATP has been claimed to have a greater role in sympathetic cotransmission in the spontaneously hypertensive rat than in the normotensive rat (Vidal et al., 1986). Whether this also holds true for hypertensive human patients remains to be established. In general, the rapid and short-lasting ATP-mediated control of vascular tone may be most appropriate in vascular beds demanding very rapid and precise adjustments.

C. NPY/Adenosine 5′-Triphosphate and Noradrenaline Cotransmission

Sympathetic neurons may thus release ATP, NA and NPY (Kasakov et al., 1988), at least at high frequency stimulation (Lundberg et al., 1994a) and this "cocktail" of messenger molecules provide a spectrum of biological actions and interactions, including different temporal information, i.e., fast, intermediate and slow signalling. The efficacy of the ATP mediated rapid and short-lasting contractions hinges on the per pulse release, but the NA-induced neurogenic contraction of blood vessels depends mainly on the intrinsic slowness and nerve activity-induced plasticity of clearance of released NA by reuptake (see Stjärne et al., 1994). For the peptide NPY, it is likely that slow removal from release sites by local enzymatic degradation and even diffusion may explain the long-lasting action, which seems to differ between vascular beds (see Lundberg and Modin, 1995).

1. Influence of adrenergic drugs: prejunctional interactions. A variety of classical drugs developed to impair sympathetic transmission influence not only NA but also NPY and/or ATP (table 5). This is often likely to be explained by transmitter costorage in the same vesicles or by coregulation of transmitter release by endogenous NA acting on prejunctional α_2-adrenoceptors. The adrenergic neuron blocker guanethidine evokes an initial release of NA with subsequent inhibition of NA secretion; it finally causes long-lasting tissue depletion of NA (Boura and Green, 1965). Guanethidine also inhibits nerve stimulation-evoked release of NPY (Lundberg et al., 1984a; Rudehill et al., 1986) and ATP (Brock and Cunnane, 1988) from sympathetic nerves; there is no initial release of these transmitters, as there is of NA, however. Guanethidine is specifically accumulated by adrenergic nerve terminals using uptake-1; then, it prevents exocytosis. Guanethidine acts as a local anesthetic by impairing conduction in adrenergic nerve terminals (Brock and Cunnane, 1988). Tyramine evokes increased resting release of NA without affecting NPY or ATP (Muramatsu, 1987; Haass et al., 1989b; Lundberg et al., 1989d).

Because the release of not only NA but also NPY (Lundberg et al., 1984a, 1989b; Dahllöf et al., 1986; Schoups et al., 1988; Haass et al., 1989a) and ATP (Stjärne, 1989) seems to be regulated via prejunctional α_2-adrenoceptors, α_2-agonists such as clonidine or UK14304 inhibit and α_2-antagonists such as yohimbine and idazoxan increase NA, NPY and ATP release. A cornerstone of sympathetic neurotransmission is thus the autoinhibition by NA acting on prejunctional α_2-adrenoceptors, thereby regulating release of all transmitters, i.e., NA, ATP and NPY (Brock et al., 1990; Starke et al., 1989). It is assumed that, for example, yohimbine potentiates neurotransmitter release by removal of ongoing α_2-autoinhibition by endogenous NA. Transmitter release to the first stimulus (i.e., single impulse stimulation) is not affected by α_2-regulation, however. Indeed, circulating plasma levels of NPY and NA are changed accordingly (increased by α_2-blockade and decreased by an α_2-agonist), even in humans, upon physiological stress activation of the sympathetic nervous system (Pernow et al., 1988c). Furthermore, treatment with α_2-agonists, such as clonidine, elevates nerve terminal content of NPY (Franco-Cereceda et al., 1987d). The finding that stimulation-evoked NPY release is markedly enhanced after reserpine treatment is also likely to be related to loss of NA-mediated α_2-adrenoceptor inhibitory regulation (Lundberg et al., 1986, 1989c, d; Modin et al., 1995). Inhibition of neuronal reuptake of NA by desipramine is associated with attenuated release of NPY (Haass et al., 1989a; Lundberg et al., 1989d) and ATP (Bao, 1993) presumably via increased biophase levels of endogenous NA activating prejunctional α_2-inhibitory mechanisms. In analogy with a parallel prejunctional regulation, P_1-purinoceptor (or rather adenosine A1-receptor, see Dalziel and Westfall, 1994) activation by adenosine analogues inhibits nerve stimulation-evoked release of ATP, NA (Sneddon et al., 1984) and NPY (Haass et al., 1989a). Furthermore, P_{2y}-purinoceptors may mediate autoinhibition of sympathetic transmitter release by ATP in both vas deferens and heart (see von Kügelgen et al., 1995).

Reserpine is a classical agent that depletes tissue monoamine stores due to interference with granular uptake (Carlsson 1965). The reserpine-induced depletion of NA is dependent on intact nerve activity during the first hours (Rosell and Sedvall, 1962) but is not, or only marginally, influenced by axotomy or ganglionic blockade after 24 hours (Lacroix et al., 1988; Pernow et

al., 1988b; Lundberg et al., 1989d). Exocytotic release of vesicle contents can still occur after reserpine treatment (Cubeddu and Weiner, 1975; Thureson-Klein et al., 1987). Moreover, ATP can still be taken up into chromaffin cell storage vesicles in spite of reserpine (Winkler et al., 1981), and ATP-mediated nerve-evoked contractions of vas deferens (Kirkpatrick and Burnstock, 1987) and isolated blood vessels remain after reserpine treatment (Muramatsu, 1987; Warland and Burnstock, 1987). The neuronal release of ATP from vas deferens is also not reduced by reserpine treatment (Kirkpatrick and Burnstock, 1987) and ATP dependent EJPs in smooth muscle cells in the vas deferens upon nerve stimulation can easily be recorded after reserpine. Interestingly, EJPs then facilitate to an even greater degree than in control tissues. Furthermore, the α_2-antagonist yohimbine has no effect on the EJPs recorded from reserpinized animals, in contrast to the large increase in amplitude of subsequent but not the first few EJPs in a train of stimuli (Brock and Cunnane, 1993; see Cunnane and Searl, 1994).

Sympathetic nerve stimulation-evoked vasoconstriction responses in a variety of vascular beds and species in vivo are often markedly reduced or even abolished in reserpinized animals (Rosell and Sedvall, 1962; Lundblad et al., 1987b; Pernow et al., 1988b). This apparent discrepancy can most likely be explained by the likelihood that a pivotal role of ATP mechanisms for sympathetic vasoconstriction is not universal and that reserpine also depletes NPY from cardiac and perivascular nerve endings (Lundberg et al., 1985c, d; Lundberg et al., 1990). Reserpine does not deplete NPY from sympathetic nerves in the vas deferens, iris, or uterus, however (Nagata et al., 1987). Furthermore, reserpine mainly depletes NPY in terminal regions of cardiovascular nerves, while levels in cell bodies and axons if anything increase due to enhanced synthesis (Lundberg et al., 1985c; Schalling et al., 1991). The depletion of NPY in cardiovascular nerves after a high dose of reserpine can be prevented by drugs or procedures that reduce sympathetic nerve impulse traffic. Thus, if reserpine is given together with the α_2-agonist clonidine (which acts centrally in low doses to reduce sympathetic tone) or with ganglionic blockers, or if preganglionic or postganglionic nerves are surgically transected, the NPY levels in the sympathetic nerve terminals are maintained. The depletion of NPY after reserpine treatment can therefore most likely be explained by enhanced NPY release in excess of peptide resupply by axonal transport (Lundberg et al., 1990). Thus, it is known that sympathetic nerve impulse traffic is increased after reserpine (Pernow et al., 1988d). Furthermore, due to the NA depletion, prejunctional α_2-adrenoceptor-mediated autoinhibition is lost. These two factors lead to a long-lasting exaggerated release of NPY and a progressive depletion of NPY in terminal regions. Furthermore, α-blockade after reserpine does not further increase NPY release (see Lundberg et al., 1990). When reserpine is combined with interruption of nerve activity, preferably by preganglionic decentralization to minimize degenerative phenomena, postganglionic stimulation leads to large, long-lasting vasoconstrictor nonadrenergic responses in a variety of vascular beds in cat, dog, and pig. Usually, these effects are mainly seen upon high frequency stimulation, although in pig nasal mucosa and skeletal muscle circulation, vasoconstrictor responses are evoked even by single impulse nerve stimulation after reserpine. Generally, the reserpine resistant vasoconstrictor effects in the pig vascular beds to high frequency stimulation are long-lasting and are thus likely to involve a peptide mediator such as NPY, which has a long half-life, but there are also rapid initial responses remaining, e.g. in skeletal muscle (fig. 10) which may be caused by a short-acting transmitter such as ATP (Lundberg and Modin, 1995; Malmström et al., 1996). Furthermore, there is good correlation between nerve-evoked NPY outflow and functional vasoconstrictor responses in these animals (Lundberg et al., 1989d; Pernow and Lundberg, 1989b; Modin et al., 1994a, 1996). The successive reduction in nerve-evoked NPY overflow upon repeated stimuli, most likely reflecting depletion of the peptide stores in the nerve terminals, has been attributed to the excessive release in relation to the limited and slow resupply of peptide by axonal transport (Lundberg et al., 1989c), even though both synthesis and axonal transport of NPY have been shown to be enhanced after reserpine (Schalling et al., 1991). In contrast, under control conditions, prejunctional α_2-adrenergic autoinhibition limits the release of NPY (as well as NA and ATP, Ramme et al., 1987), and the supply of NPY in the nerve terminal is then not easily depleted (Modin et al., 1994a).

The recent experiments using the Y_1 receptor antagonist BIBP 3226 have revealed that endogenous NPY is likely to contribute to the long-lasting phase of the vasoconstriction seen upon high frequency sympathetic stimulation in hind limb, nasal mucosa and skin of control pigs (Lundberg and Modin, 1995). Furthermore, after reserpine treatment, i.e., in the absence of NA, BIBP 3226 and SR120107A markedly reduced the duration of the vasoconstriction in response to high frequency stimulation in all vascular beds studied, whereas the peak amplitude of the vascular responses was attenuated only in kidney and spleen (fig. 10). As expected, the vasoconstrictor response to single impulse stimulation was largely uninfluenced by BIBP 3226 and SR120107A (Lundberg and Modin, 1995; Malmström et al., 1995). Therefore, in pig hind limb and nasal mucosa both the nonadrenergic response to single impulses and the peak effect upon high frequency stimulation after Y_1 receptor blockade may involve some rapidly acting and quickly metabolized transmitter, such as ATP (von Kügelgen and Starke, 1985; Muramatsu, 1987; Bulloch and McGrath, 1988a, b) (fig. 10). Because the release of ATP is normally inhibited by endogenous NA acting on

prejunctional α_2-adrenoceptors (Ramme et al., 1987), also the purine release upon high frequency burst stimulation is likely to be increased after reserpine (in contrast to single impulse stimulation, see Brock et al., 1990). In the rat, α,β-methylene ATP tachyphylaxis reduces the vasopressor response to sympathetic nerve stimulation in vivo (Bulloch and McGrath, 1988a). Unfortunately, in vivo desensitization with a P_{2x} receptor agonist in the pig has not yielded conclusive evidence for ATP being a sympathetic transmitter of reserpine-resistant vasoconstriction in nasal mucosa (Lacroix et al., 1988, 1989) and spleen (Lundberg et al., 1989d) due to interference with NPY overflow. Also suramine exerts complex actions on both P_{2x} and P_{2y} receptors because there is in vitro evidence that ATP acts presynaptically to inhibit neurotransmitter release via P_{2y} receptors (von Kügelgen et al., 1989; see von Kügelgen et al., 1995). The rapid remaining vasoconstriction to sympathetic nerve stimulation in pig nasal mucosa after reserpine treatment (to deplete NA) and Y_1 receptor antagonist treatment was uninfluenced by suramine, although the effect of exogenous α,β-methylene ATP was blocked (Malmström et al., 1996). Further experiments using more selective P_{2x}- and P_{2y}-receptor antagonists will be necessary to establish the role of ATP as a sympathetic transmitter in various vascular beds and other organs in vivo.

2. Postjunctional interactions. ATP, NA, and NPY represent three types of mediators with specific receptor mechanisms and clearly different time spans of action, ATP acting on P_{2x} receptors being a fast ionotropic transmitter (milliseconds), NA a medium transmitter (seconds, duration depending both on influx of Ca^{2+} and release of Ca^{2+} from intracellular stores), and NPY a long-acting transmitter (minutes, depending on adenylyl cyclase inhibition and Ca^{2+} mobilization mechanisms). A variety of postjunctional synergistic interactions have been reported to occur between these agents: for example, NPY enhances NA- (Ekblad et al., 1984; Pernow et al., 1986a) and ATP- (Saville et al., 1990) evoked contractions of vas deferens (Stjärne et al., 1986) and blood vessels. Potentiating effects of NPY are prevented by removal of extracellular Ca^{2+}, are partially prevented by an L-type Ca^{2+} channel blocker (Pernow et al., 1986a) and are mimicked by a Ca^{2+} channel activator. Pharmacological modulation of adenylyl cyclase had no effect, however (Cressier et al., 1995). Both direct and indirect (potentiating) vascular effects of NPY are mediated via Y_1 receptors. The accompanying rise in inositolphosphate may be secondary to an increase in intracellular Ca^{2+} (Cressier et al., 1995). The exact mechanisms underlying these interactions remain to be established, but in analogy with the situation for α_1- and α_2-receptors (McGrath et al., 1991), cooperative interactions may occur downstream of the receptor, when several types of mechanisms converge to increase intracellular Ca^{2+}: voltage-gated Ca^{2+} channels (ATP, P_{2X1} receptors), receptor-operated Ca^{2+} channels (NA, α_1- and α_2- receptors), release of Ca^{2+} from intracellular stores via the IP_3 system, and reduced activity of adenylyl cyclase (NPY, α_2-receptor).

In addition to short term contractile events, there is evidence that NPY, NA and ATP interact synergistically to stimulate proliferation of human vascular smooth muscle cells (Erlinge et al., 1994).

V. Conclusions, Emerging Principles and Future Perspectives

As has been shown in this review, transmission in the autonomic nervous system is exceedingly complex. The chemical signaling in autonomic sensory and motor neurotransmission involves a variety of agents with different characteristic patterns of synthesis, storage, release, receptors and inactivation. Large species and tissue variations also occur but, in several cases, it seems clear that these different combinations of transmitters may reflect specialized functions. Even if a certain transmitter response is not evident in healthy human individuals, it may be of clinical relevance, as disease states may markedly change receptor expression, transmitter synthesis or degradation, etc. Furthermore, the various animal models that are cornerstones in the basic pharmaceutical development process need to be characterized with respect to the transmitter mechanisms involved. The recent developments in gene knock-out technology will make the mouse increasingly important as an experimental animal. This technique may help us deduce the relative importance of individual peptides for the transmission process, even if these act on multiple receptors. Several basic principles of transmission on the autonomic nervous system are emerging. Thus, specific combinations of chemical signals constitute cocktails of released transmitters, which vary in composition depending on e.g., strength and duration of activation whereby peptide release generally requires strong activation (except for sensory nerves). The chemical signals that presently fulfill transmitter criteria, i.e., NA, ACh, ATP, glutamate, NO and certain neuropeptides, are distinctly different with regard to time span of action: ATP, ACh and glutamate are the most short-lived, and can represent ionotropic transmitters, whereas monoamines and neuropeptides, such as tachykinins and NPY, act on G-protein coupled receptors and have a longer duration of action. This is especially true of the peptides which, due to slow degradation, have a very long duration of action that in part may compensate for more restricted release. Monoamines generally bind in pockets between the transmembrane receptor segments, while peptides bind in several modes with major interaction sites in the extracellular part of the receptors. NO has, in contrast to other transmitters, no specific storage mechanisms or any receptor in the plasmalemma but diffuses and binds tightly to its target effector, intracellular guanylyl cyclase, and possibly other enzymes and ion channels. It is

still a matter of speculation why these different chemical signals are stored and produced sometimes separately, sometimes in the same neuronal varicosities, but the transmitters usually serve complementary and synergistic functions, as can easily be shown regarding control of vascular smooth muscle. There are also instances in which the released transmitters clearly have opposing functions, for example CGRP and TKs, which are coreleased from sensory nerves in urogenital smooth muscle. The resulting response depends on degree of nerve activation (amount released), peptide degradation and receptor localization. Even if Dale's principle (Dale, 1935) states that a mature neuron should make use of the same transmitter at all of its synapses (or release sites from varicosities), it is evident that the bulk of the sensory neuropeptides are transported into the peripheral rather than in the central branch. Thus, the transmitter function of TKs and CGRP in a variety of reactions seen upon activation of sensory nerves in the periphery has now been well established. In contrast, a peripheral role for glutamate, the main transmitter for the central branch of the sensory neurons, remains to be established.

Classical drugs that interfere with autonomic transmission, especially at prejunctional receptors regulating release, often have profound effects on not one but several transmitter mechanisms including those of NA, ACh, ATP and neuropeptides, leading to changed mediator contribution compared with the control situation. The development of novel specific receptor antagonists has helped us to define some of the roles of individual ingredients in the transmission cocktail that is released upon neuronal activation. However, there is still need for development of nonpeptide receptor antagonists to VIP, CGRP and NPY receptors as well as P_{2x} receptor antagonists and selective neuronal NOS inhibitors. Monoamine antagonists often bind close to where the agonist binds (isosterically) to the receptor. Nonpeptide antagonists for peptide receptors, on the other hand, often bind at different sites from the peptide agonist (allosterically) and may act by stabilizing inactive conformations of the receptors. These new agents, including peptide receptor antagonists and agonists of nonpeptide nature, may in time point the way to novel pharmaceuticals for treatment of human disease states involving autonomic function as well as sensory transmission. Possibly, molecular modeling of small nonpeptide antagonists and agonists combined with receptor mutagenesis can resolve the architecture of the active receptor site and identify the actual chemical interaction between a chemical moiety of the ligand and a particular amino acid of the receptor: this could lead to a future rational design of novel drugs. Finally, principles of neurotransmission that were originally characterized in the peripheral nervous system due to easily accessible experimental models are likely to have major impact also on our understanding of brain function in normal conditions and disease states.

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