Université de Montréal

Functional implication of L-type Ca^{2+} channel in catecholamine secretion induced by PACAP in canine adrenal gland in vivo

par

Guoju Geng

Faculté de pharmacie

Mémoire présenté à la Faculté des études supérieures

en vue de l'obtention du grade de

Maîtrise ès sciences (M.Sc.)

en pharmacodynamie biochimique

November 1997

Université de Montréal

Faculté des études supérieures

Ce mémoire intitulé:

Functional implication of L-type Ca^{2+} channel in catecholamine secretion induced by PACAP in canine adrenal gland in vivo

présentée par

Guoju Geng

a été évaluée par un jury composé des personnes suivantes:

Président-rapporteur: Dr. Huy Ong

Directeur de recherche: Dr. Nobuharu Yamaguchi

Membre du jury: Dr. Daniel Lamontagne

Mémoire accepté le: 20. 04. 1998

SOMMAIRE

Titre:

Implication fonctionnelle de canaux Ca^{2+} dans la sécrétion des catécholamines de la glande surrénales induites par PACAP chez le chien in vivo.

Introduction:

Le polypeptide hypophysaire activateur de l'adenylate cyclase ("pituitary adenylate cyclase activating polypeptide", PACAP) a été suggéré comme étant un neurotransmetteur non-cholinergique qui stimule la libération des catécholamines sécrétées par les cellules chromaffines chez le rat. Le PACAP semble à la fois augmenter l'entrée du calcium (Ca^{2+}) extracellulaire et la libération du Ca^{2+} intra-cellulaire des cellules chromaffines. La libération des catécholamines des cellules chromaffines en culture induite par le PACAP serait probablement bloquée par un bloqueur des canaux Ca^{2+} de type-L, suggérant que ces canaux jouent un rôle important sur la sécrétion des catécholamines. Malgré le fait que les canaux Ca^{2+} de type-L (sensibles aux dihydropyridines) ont été impliqués dans la libération des catécholamines induite par le PACAP sous les conditions in vitro, leurs rôles fonctionnels demeurent inconnu in vivo.

Buts:

Nous allons tenter d'étudier l'implication fonctionnelle d'un mécanisme régulateur potentiel sur la libération des catécholamines induite par le PACAP in vivo. Pour ce faire, nous allons examiner la libération des catécholamines en réponse au PACAP en présence de nifédipine (un bloqueur des canaux Ca^{2+} de type-L) chez le chien anesthésié. Le but spécifique de cette étude est de vérifier, en premier lieu, si les deux isomères du PACAP (-

27 et -38) stimulent directement la sécrétion des catécholamines, et également de montrer l'implication possible des canaux Ca^{2+} de type-L dans cette réponse hormonale.

Méthodes:

Des chiens adultes étaient anesthésiés avec du pentobarbital sodique à une dose initiale de 30 mg/kg, iv. Les médicaments ont été administrés localement dans l'artère lombo-surrénale gauche. Les concentrations plasmatiques d'épinephrine et de norépinephrine ont été déterminées dans le sang veineux surrénal lien gauche et le sang aortique par l'HPLC avec détecteur électrochimique.

Résultats:

L'administration locale du PACAP27 (n = 11) ou PACAP38 (n = 7) (3.2, 32 and 320 nM) a augmenté la sécrétion basale des catécholamines surrénaliennes de manière dose-dépendante. La réponse des catécholamines induite par le PACAP27 était 3 à 5 fois supérieure à celle du PACAP38 selon les doses utilisées. Dans le groupe contrôle, le PACAP27 (50 µg, 32 nM) en présence du véhicule (6% ethanol) a augmenté de façon reproductible la sécrétion basale des catécholamines à la seconde administration de la même dose du PACAP27. Dans le groupe recevant la nifédipine (50 µg, 289 µM), l'augmentation nette des catécholamines en réponse au PACAP27 a été significativement inhibée d'environ 50% (n = 9). Cependant, dans des conditions similaires, une augmentation de la sécrétion basale des catécholamines induite par BAY-K 8644 (5 µg, 2.9 µm) (un activateur des canaux Ca^{2+} de type-L), a été complètement inhibée en presence de la nifédipine (n = 6).

Conclusion:

Les PACAP27 et PACAP38 augmente la sécrétion basale des catécholamines

surrénaliennes de façon dose-dépendante par leur action directe au niveau de la médullo-surrénale chez le chien in vivo. La présente étude suggère que cette réponse serait médié en partie par les canaux Ca^{2+} de type-L chez le chien anesthésié.

SUMMARY

Background:

Pituitary adenylate cyclase-activating polypeptide (PACAP) has been suggested as a non-cholinergic neurotransmitter to stimulate catecholamine secretion from rat chromaffin cells. PACAP has been shown to increase Ca^{2+} influx and Ca^{2+} release from intracellular sources in chromaffin cells. PACAP-stimulated catecholamine release in cultured adrenal medullary cells is likely to be blocked by an L-type Ca^{2+} channel blocker, suggesting that such channels play an important role in hormone secretion. Although dihydropyridine-sensitive L-type Ca^{2+} channels have been implicated in PACAP-induced catecholamine release in vitro, their functional role still remains unclear under in vivo conditions.

Objectives:

We therefore attempted to demonstrate the functional significance of L-type Ca^{2+} channels potentially involved in PACAP-induced catecholamine secretion in vivo. To this end, adrenal catecholamine release in response to PACAP was examined in the presence of nifedipine (a dihydropyridine sensitive L-type Ca^{2+} channel blocker) locally administered to the adrenal gland in anesthetized dogs. The specific aim of the present study was to investigate whether two isomers of PACAP, consisted of 27 (PACAP27) and 38 (PACAP38) amino acid residues, directly stimulate adrenal catecholamine release and whether L-type Ca^{2+} channels are involved in PACAP27-induced adrenal catecholamine secretion in anesthetized dogs.

Methods:

Adult mongrel dogs were anesthetized with pentobarbital sodium (30 mg/kg, iv).

Drugs were locally infused into the left adrenal gland through the left adrenolumbar artery. Plasma epinephrine and norepinephrine concentrations in adrenal venous and aortic blood were determined by an HPLC with electrochemical detection.

Results:

Local administration of either PACAP27 (n = 11) or PACAP38 (n = 7) resulted in an increase in catecholamine output in a dose-dependent manner ($P< 0.05$) with doses ranging from 3.2, 32 to 320 nM. The catecholamine response to PACAP27 were ~ 3 to 5 times greater than those to PACAP38 within the dose range tested. In the control group, receiving the vehicle (6% ethanol), PACAP27-induced catecholamine secretion was highly reproducible upon the repeated administration of PACAP27 (50 μg, 32 nM). In dogs receiving nifedipine (50 μg, 289 μM) similarly administered to the left adrenal 5 min before PACAP27 infusion, the net increase in adrenal catecholamine response to PACAP27 was significantly inhibited by 50% (n = 9). However, under similar experimental conditions, the adrenal catecholamine response to BAY-K 8644 (5 μg, 2.9 μM) (an L-type Ca^{2+} channel activator) was completely abolished in the presence of the same dose of nifedipine (n = 6).

Conclusions:

Both PACAP27 and PACAP38 increase adrenal catecholamine secretion in a dose-dependent manner by their direct action on the medulla of canine adrenal gland in vivo. The study suggests that PACAP-induced adrenal catecholamine release is, at least in part, mediated by the dihydropyridine-sensitive L-type Ca^{2+} channels in anesthetized dogs.

TABLE OF CONTENTS

LIST OF TABLE

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS AND SYMBOLS

α	alpha
β	beta
ACh	acetylcholine
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
Ca^{2+}	calcium
CA	catecholamine
COMT	catechol-O-methyltransferase
DA	dopamine
DBH	dopamine β-hydroxylase
DMPP	1,1-Dimethyl-4-phenylpiperazinium
DOMA	3-methoxy- 4-hydroxymandelic acid
EK	enkephalins
HPLC	high performance liquid chromatography
HVA	homovanillic acid
IP3	inositol-1, 4, 5-triphosphate
iv	intravenous
MAO	monoamine oxidase
MHPG	3-methoxy-4-hydroxyphenylgycol
N	number

Na^+	sodium
NIF	nifedipine
NPY	neuropeptide Y
NSF	N-ethylmaleimide-sensitive fusion protein
PACAP	pituitary adenylate cyclase activating polypeptide
PACAP-IR	pituitary adenylate cyclase activating polypeptide immunoreactive
PACAP-LI	pituitary adenylate cyclase activating polypeptide like immunoreactive
PI	phosphatidylinositol
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PNMT	phenylethanolamine N-methyl transferase
RIA	radioimmunoassay
SNAP	synaptosome associated protein
SNAP-25	synaptosome associated protein of 25kDa
VAMP	vesicle associated membrane protein
VMA	vanillylmandelic acid (3-methoxy-4-hydroxymandelic acid)
VIP	vasoactive intestinal polypeptide

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor, Prof. Nobuharu Yamaguchi, for his uninterrupted devotion and patience during my studies. His high scientific standard and mastery of physio-pharmacology in "*in vivo*" modelling has given me an opportunity to expand my knowledge in the field. His keen insights and constructive criticisms have been a great source of encouragement and guidance.

I would like to thank Dr. Robert Goyer, the Dean of Faculty of Pharmacy, for providing me with this opportunity to study and all the members of the Faculty of Pharmacy for their assistance during my study.

I am very grateful to Dr. Daniel Lamontagne for his persistent and patient assistance with my research. I would also like to express my appreciation to my colleagues Daniel Martineau, Fethi Trabelsi, Rania Gaspo and Stéphane Lamouche for their valuable opinions and friendship during the past two years.

I would like to thank Sanae Yamaguchi, Richard Lalande and Yuka Fukushima for their valuable technical assistance in surgical preparations as well as in various experimental protocols.

A studentship (1996-1997) from the Group de recherche sur le système nerveux autonome (GRSNA) of the Université de Montréal and a financial support from Prof. N. Yamaguchi (1995-1997) are gratefully acknowledged.

I want to thank my family for their patience, support and love without which the completion of this dissertation would not have been possible.

CHAPTER 1

INTRODUCTION

1.1. BRIEF HISTORY OF NEUROPEPTIDE

The concept of chemical neurotransmission goes back to the beginning of this century, but was confined to the peripheral nervous system for a long time. According to the observation that the adrenal extract mimicked the effect of epinephrine, Elliot suggested, in 1905, that epinephrine might be released from sympathetic nerves. The hypothesis that an epinephrine-like substance might be released from sympathetic nerve endings has thus been suggested, but it remained unproven until Loewi demonstrated the first direct evidence that a chemical transmission is opsrative in the peripheral neuro-effector junction (Loewi 1921). Loewi found that the perfusion fluid obtained from the first isolated, perfused frog heart receiving electrical stimulation of the vagus nerve could decelerate the second heart similarly perfused but without nerve stimulation (Loewi 1921). At the same time, Cannon and Uridil demonstrated that an epinephrine-like substance was obtained during hepatic sympathetic nerve stimulation resulting in increase in blood pressure and heart rate (Cannon and Uridil 1921). However, it was wrongly assumed until 1946 when Von Euler demonstrated that norepinephrine is a sympathetic neurotransmitter (Von Euler 1946a, b, c). In 1949, Peart reported the finding that norepinephrine was released from the spleen when sympathetic nerves are stimulated, establishing that norepinephrine is the neurotransmitter released from sympathetic nerve endings (Peart 1949), by contrast to epinephrine which is the major catecholamine released from the adrenal medulla (Cannon and Rosenbleuth 1937).

The term neurohumoral has been used to explain the observations relating to the transmission of impulses from postganglionic autonomic fibers to effector cells. The term "peptidergic neuron" was first introduced by Bargmann et al (1967), as the synapse-like terminals on endocrine epithelial cells of the cat hypophysis were discovered. Many novel peptides in the central nerve system have been discovered in 1980s. In addition, immunobiological tests have shown that neuropeptides are widely distributed in mammalian and unicellular organisms (Roth and Le Roith 1984). According to the recent new definition, any compound consisting of 100 or fewer amino acids can be considered as a peptide. Polypeptide consists of 100-200 amino acids, and proteins consists more than 200 amino acids (Kastin et al. 1996).

Since norepinephrine has been demonstrated as a neurotransmitter, it was generally assumed that each nerve cell could synthesize and release only one neurotransmitter (Eccles et al. 1954). More recently, it is generally accepted that a nerve cell can produce more than one transmitter (Leonard 1984). It has been found that not only two components of the same peptide (e.g., neuropeptide Y [NPY] and its C-terminal flanking peptide) can be colocalized, but also more than one peptide can be stored with a classical neurotransmitter (Gulbenkian et al. 1985). Moreover, many polypeptide hormones which were recently discovered have been suggested to act as neurotransmitters or neuromodulators (Sundler et al. 1985). Neuropeptides have been extensively studied in the central and the peripheral autonomic nervous system. Neuropeptides are chemical messengers and their major role seems to be the local modulation of amine and amino acid neurotransmission (Hughes and

Woodruff 1992).

Substance P, discovered by Von Euler and Gaddum (1931), has been chemically identified by Leeman (1980) and found to be a multifunctional neuropeptide. It has been shown that the substance P exist in splanchnic nerve and in nerve terminals in the adrenal medulla (Livett and Marley 1993). This suggests that the substance P may play a role in modulating the cholinergic secretion of adrenal catecholamines from the adrenal medulla.

Among various peptides so far identified, angiotensin II is the peptide in that it's amino acid sequence was first established in 1945 (Sundler et al. 1985). It was found that angiotensin II releases adrenal catecholamine more than 50 years ago (Braun-Menendez et al. 1940). In 1958, Hillarp found the protein composition of adrenal chromaffin granules. The development of immunohistochemical techniques has led to precisely localize some of the neuropeptides within chromaffin cells in the adrenal medulla and/or sympathetic nerve fibres in several species.

Said and Mutt first isolated vasoactive intestinal polypeptide (VIP) from porcine intestine in 1970 (Said and Muttt 1970). VIP, composed of 28 amino acids, is known to be a circulating hormone under physiological conditions. It is also well documented that VIP plays a role of neurotransmitter (Fahrenkrug 1979, 1991) and is implicated in a peptidergic stimulatory mechanism of adrenal medullary catecholamine secretion (Houchi et al. 1987). In 1989, pituitary adenylate cyclase activating polypeptide (PACAP), a neuropeptide

belonging to the VIP/secretion/glucagon family, was detected in ovine hypothalamic tissues based on its ability to stimulate adenylate cyclase activity in cultured pituitary cells (Miyata et al. 1989). It has been suggested that PACAP is a potent noncholinergic secretagogue for catecholamines and also functions as a neurotransmitter, neuromodulator, and neurotrophic factor in the central nervous system (Arimura and Shioda 1995).

It has become increasingly clear that various neurochemical messengers are co-existing in a variety of nerves, both in the brain and peripheral tissues (Lundberg and Hökfelt 1983). There is some speculation. Such as conventional neuroregulators may function as primary neurotransmitters, and peptides may function as "cotransmitters" in an auxiliary capacity of the same neuron. More recently, there have been a lot of newly discovered neuropeptides. Among those neuropeptides, VIP (Bloom and Polsk 1980), NPY (Tatemoto et al. 1982) and galanin (Dunning et al. 1990) and endothelin (Yanagisawa et al. 1988) may be the most well characterized peptides functionally involved in the peripheral autonomic nervous system. More recent studies indicate that PACAP is likely to be involved in the local regulation of chromaffin cell functions in vitro (Watanabe et al. 1992, Wakade et al. 1992, Isobe et al. 1993), although its functional implication remains to be determined under in vivo condition.

1.2. SYMPATHOADRENAL SYSTEM

The autonomic nervous system is organized on the basis of reflex arc, and consists

of afferent, central and efferent elements. Impulses initiated in visceral receptors are relayed via afferent autonomic pathway to the central nervous system, integrated within it at various levels, and transmitted via efferent pathways to visceral effectors. The efferent autonomic nervous signals are transmitted to the body through the sympathetic and parasympathetic nervous systems, which have the functions in regulating the internal environment of the organism. The sympathoadrenal system includes two components. One is the sympathetic nervous system that releases the neurotransmitter norepinephrine from its postganglionic neurons. Another is the chromaffin tissues, including the adrenal medulla that secrete epinephrine, norepinephrine, and dopamine (Shah et al. 1984, Landsberg and Young 1985). The organization of the sympathoadrenal system is shown in Fig. 1.1. The regulation of physiological functions by catecholamines is mediated by both sympathetic nerves and the adrenal medulla.

1.2.1 Sympathetic nervous systems

The sympathetic nervous systems are located in the thoracic and upper lumber segments of the spinal cord. The preganglionic neurons in the intermediate cell column of the spinal cord, which receive neuronal inputs directly from several regions of the central nervous system and innervate the postganglionic sympathetic neurons in the paravertebral and preaortic sympathetic ganglia. The axons of the preganglionic neurons are originated between T-1 and L-5, which may synapse on postganglionic neurons in this ganglion, or they may pass through the ganglion and enter either the sympathetic chain or a splanchnic nerve. The postganglionic axon passes through a grey communicating ramus back to the

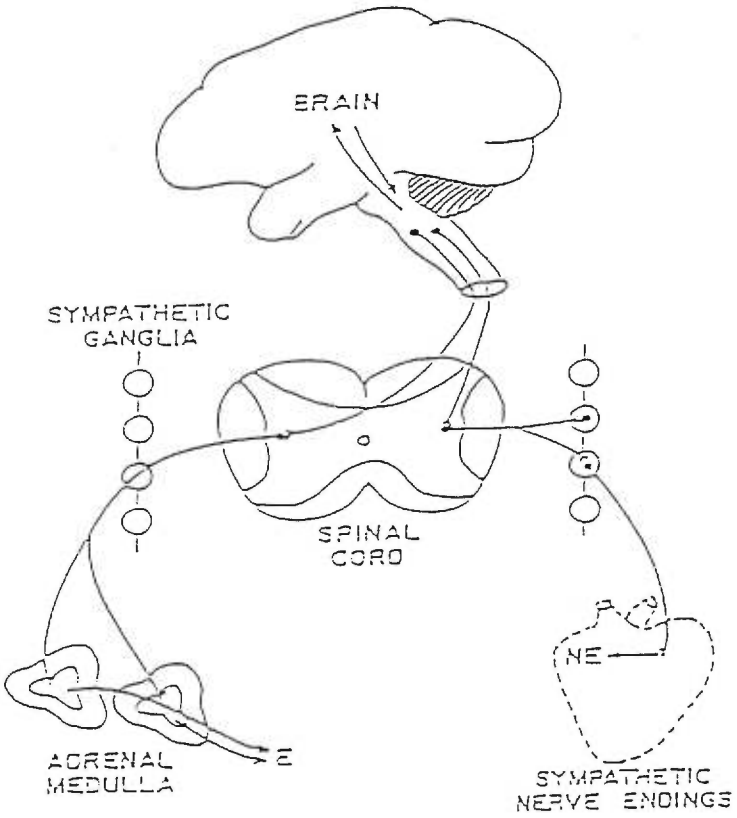

Fig. 1.1 Organization of the sympathoadrenal system. Descending tracks from the medulla oblongata, pons, and hypothalamus synapse with preganglionic sympathetic neurons in the spinal cord. Preganglionic neurons, in turn, innervate adrenal medulla directly or synapse in paravertebral ganglia with postganglionic sympathetic neurons. The latter gives rise to sympathetic nerves, which are distributed widely to viscera and blood vessels. Release of epinephrine or norepinephrine at the adrenal medulla or sympathetic nerve endings occurs in response to a downward flow of nerve impulses from regulatory centres in the brain. (From Landsberg and Young. 1985)

spinal nerve for distribution to sweat glands and pilomotor muscles, and to blood vessels of skeletal muscle and skin.

The preganglionic sympathetic neurons are cholinergic. In contrast, however, most of the postganglionic sympathetic neurons are adrenergic. The peripheral sympathetic nerve endings synthesize and store norepinephrine and release the stored norepinephrine in response to sympathetic nerve impulses. The nerve endings also take up catecholamines from the extracelluar fluid. Some preganglionic axons pass through a splanchnic nerve and end directly into the adrenal medullae. There, they end directly on modified neuronal cells (chromaffin cells) that secrete epinephrine and norepinephrine into the blood stream (Goodman and Gilman 1996).

1.2.2 Adrenal medulla

The adrenal medulla is a sympathoendocrine organ that embryonically and anatomically is similar to sympathetic ganglia; all of them are derived from the neural crest. In mammals, the adrenal medulla is composed of chromaffin cells surrounded by adrenal cortex. These chromaffin cells contain large numbers of vesicles or granules, which are important in the storage and secretion of catecholamines. The adrenal medulla contain distinct epinephrine- and norepinephrine-containing cells distinguishable on the basis of differences in the electron microscopic appearance of chromaffin granules as well as differences in biochemical composition (Kryvi 1979). The cells are surrounded by nerves, connective tissue, and blood vessels (Perlman and Chalfie 1977).

The cells of the adrenal gland receive both an intrinsic and an extrinsic innervation. The external fibres reach the adrenal gland via the splanchnic nerves. The chromaffin cells in the adrenal medulla receive a preganglionic sympathetic fibers, which release acetylcholine (ACh) at the synapses. ACh stimulates catecholamine secretion from adrenal medullary chromaffin cells. Recently, it has been shown that the medulla is innervated by both pre- and postganglionic sympathetic and parasympathetic fibers (Breslow 1992, Parker et al. 1993) as well as afferent sensory nerves (Breslow 1992, Parker et al. 1993). In addition, peptidergic nerves are also present in these ganglion cells. Met- and leu-enkephalin, NPY and VIP immunoreactive neuropeptides have also been localized in the adrenal gland (Breslow 1992, Parker et al. 1993). Furthermore, PACAP immunoreactivity has been found in the rat adrenal medulla (Tabarin et al. 1994, Frödin et al. 1995).

The adrenal medulla and the sympathetic postganglionic neurons share a variety of physiological processes including the synthesis, storage, and release of catecholamines. The catecholamines serve both as neurotransmitters and hormones.

1.3. BIOCHEMISTRY OF CATECHOLAMINES

The endogenous catecholamines include epinephrine (adrenaline), norepinephrine (noradrenaline) and dopamine. Epinephrine is a neurotransmitter in certain selected regions of the central nervous system. Norepinephrine is the peripheral and central adrenergic

neurotransmitter. Dopamine is a neurotransmitter in the central nervous system, but its functional role in periphery remains unclear.

1.3.1 Chemistry, site of formation and synthesis of catecholamines

The basic chemical reaction of catecholamine synthesis is shown in Fig. 1.2. The sequence of catecholamine synthesis is as follows: tyrosine → dihydroxyphenylalanine → dopamine → norepinephrine → epinephrine. Catecholamines are synthesized from the amino acid tyrosine, which enters neurons and chromaffin cells via an active transport mechanism and is converted to dihydroxyphenylalanine (dopa). This reaction is catalyzed by tyrosine hydroxylase, which appears to be in the cytosol of both adrenal medulla and sympathetic nerve ending (Landsberg and Young 1985). The conversion of dopa to dopamine is catalyzed by the enzyme aromatic L-amino acid decarboxylase (dopa decarboxylase), which can be found in all the tissues. The dopamine formed in the cytoplasm must be taken up by the chromaffin granule before it can be processed further. The conversion of dopamine to norepinephrine is catalyzed by dopamine β-hydroxylase (DBH) which is present exclusively within the granules (Winkler et al. 1986). Synthesis of norepinephrine begins inside the vesicles in the axoplasm of the terminal nerve endings of adrenergic nerve fibres (Fig. 1.3).

In the adrenal medulla, this reaction goes still one step further to transform about 80 per cent of the norepinephrine into epinephrine (Fig. 1.4). Most of the norepinephrine leaves the granules again and passes into the cytoplasm. Phenylethanolamine N-methyl transferase (PNMT) catalyzes the N-methylation of norepinephrine to epinephrine, using S-

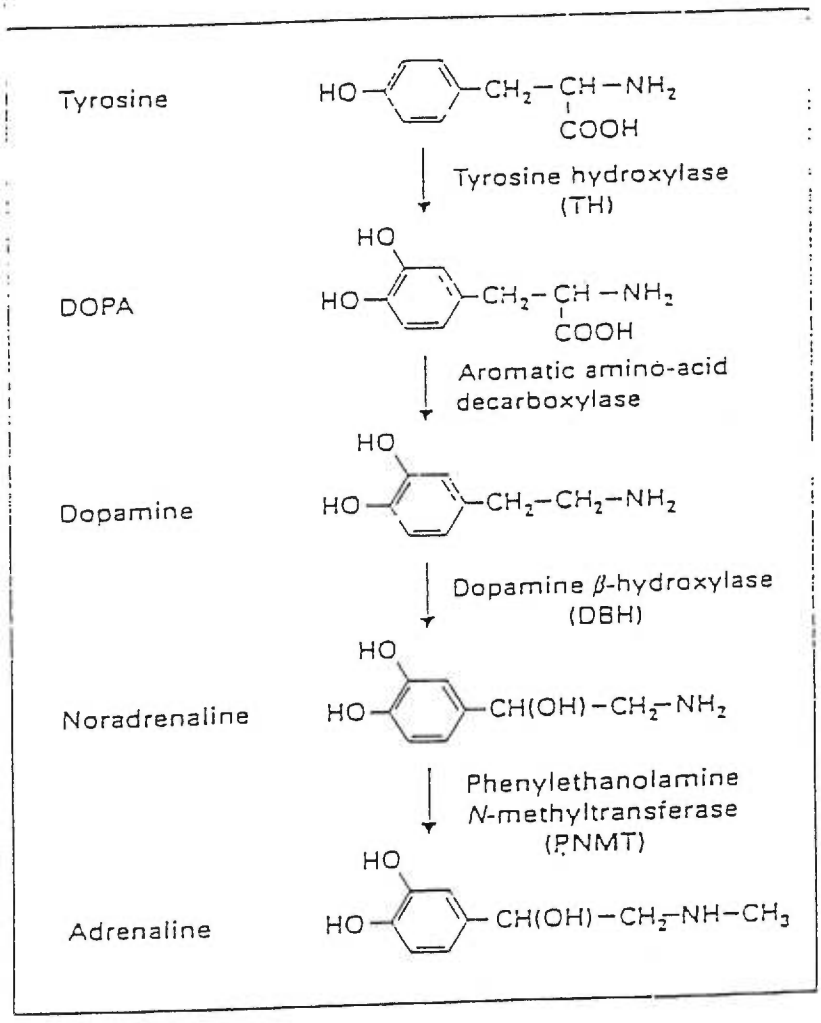

Fig. 1.2 Biosynthetic pathway of norepinephrine (noradrenaline) and epinephrine (adrenaline) in the adrenal medulla. Four enzymes are involved: tyrosine hydroxylase (TH), aromatic amino-acid decarboxylase (dopa decarboxylase, DDC), dopamine β-hydroxylase (DBH) and phenylethanolamine N-methyltransferase (PNMT). (From Cryer 1992).

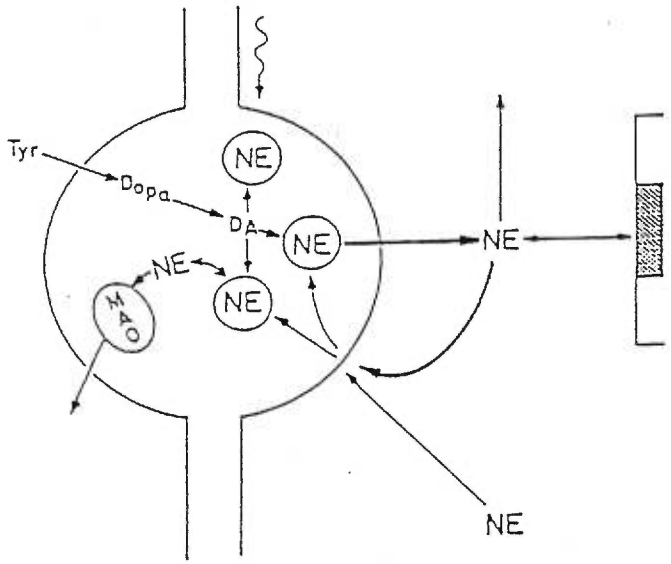

Fig. 1.3 Schematic representation of sympathetic nerve ending. Tyrosine is taken up by the neuron and sequentially converted to dopa and DA; after uptake into the granule, DA is converted to NE. In response to nerve stimulation, NE is released into synaptic cleft, where it may diffuse into circulation. (From Landsberg and Young 1985).

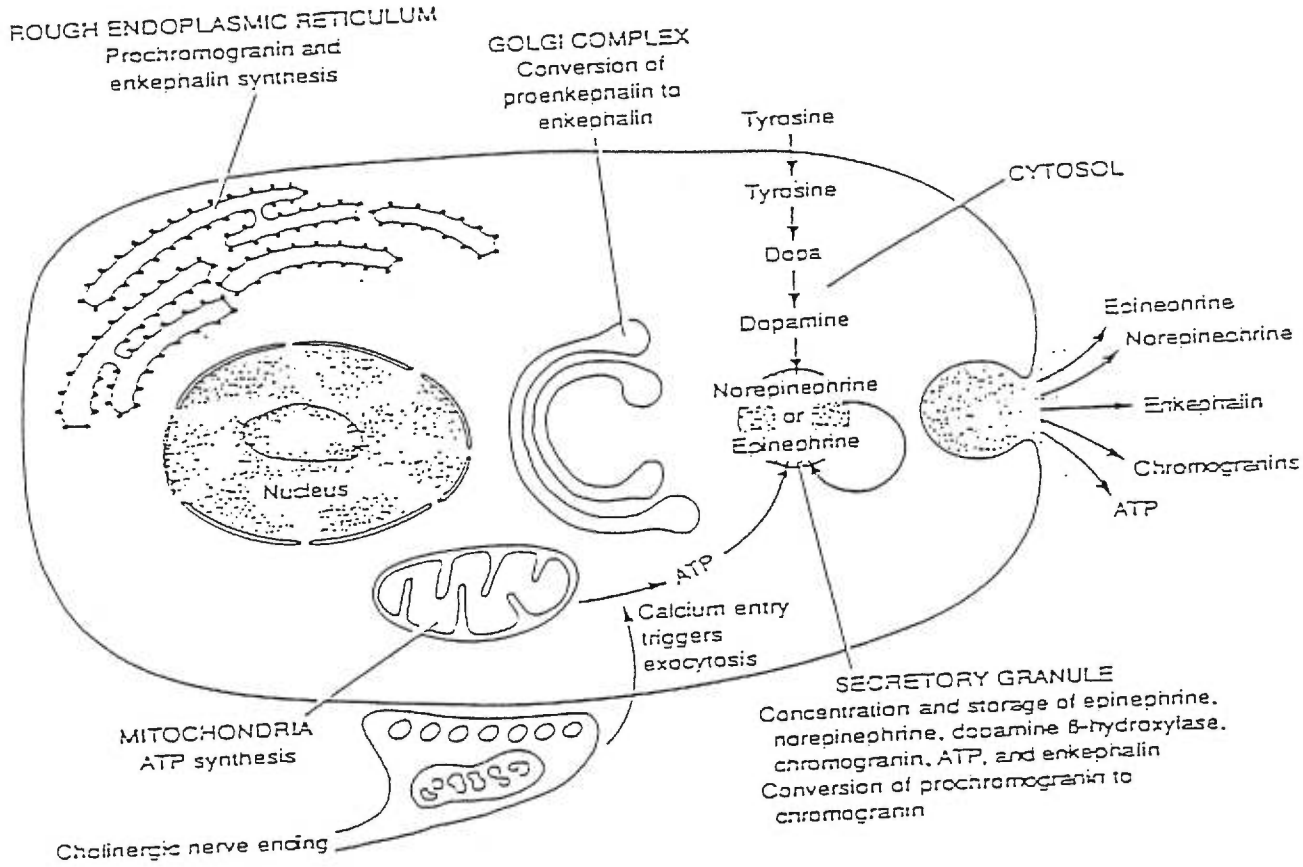

Fig. 1.4 Diagram of an adrenal medullary cell showing the role of several organelles in synthesizing the constituents of secretory granules. Norepinephrine synthesis takes place in granules and its conversion to epinephrine in the cytosol (From Junqueira et al. 1992).

adenosylmethionine as the methyl donor. Epinephrine thus formed is stored again in the granules. PNMT appears in the cytosol in the epinephrine-containing cells of the adrenal gland (Coupland 1972) and a few neurons in the central nervous system that utilizes epinephrine as a neurotransmitter (Saavedra et al. 1976).

1.3.2 Storage of catecholamines

Catecholamines are stored within specific intracellular electron-dense granules of the adrenal medulla and of the sympathetic neurons, and appear in physiologically inactive form as long as they are within granules. The chromaffin granules contains, by dry weight: catecholamines (21%), proteins (35%), lipids (22%), and adenosine triphosphate (ATP) (15%) (Winkler et al. 1986). Calcium and ascorbic acid are present in high concentrations within the granule (Stjarne 1972). DBH is one of the proteins in the chromaffin granules, which synthesizes norepinephrine from dopamine (Johnson 1988). Norepinephrine and its methyl derivative, epinephrine, are secreted by the adrenal medulla, but epinephrine is not a mediator at postganglionic sympathetic endings. It is interesting that some neuropeptides such as NPY and galanin have been identified in chromaffin granules (Majane et al. 1985, Bauer et al. 1986).

Norepinephrine is localized in the storage granules of the sympathetic nerve ending. Though granules in the sympathetic nerve endings are similar to those in the adrenal medulla in appearance, it is considerably small in size and contain only norepinephrine is found throughout the entire sympathetic nervous system. The granules containing norepinephrine,

can absorb and inactivate circulating norepinephrine (Stjärne 1966). The vesicles of peripheral sympathetic nerves uptake dopamine (DA) from the cytosol by an active transport mechanism, and covert it to norepinephrine, and store norepinephrine in the form of intravesicular complex involving ATP (Smith 1972). As in the case of adrenal medullary chromaffin granules, the large vesicles in the sympathetic nerve ending contain ATP, DBH, and neuropeptides such as NPY (Millhorn and Hokfelt 1988, Winkler et al. 1987) and substance P (Kessler et al. 1983), which are released in response to nerve impulses.

1.3.3 Release of catecholamines

Catecholamines can be released from the granules by physical, chemical, and neural means. Catecholamine release at the sympathetic nerve endings and adrenal medulla is under the direct and exclusive control of the central nervous system. In the adrenal medulla, the physiological stimulus for adrenal catecholamine secretion is the liberation of ACh by the preganglionic fibres of the splanchnic nerves. The interaction of ACh with nicotinic receptors is to activate receptor-associated cation channels in the chromaffin cell, which in turn leads to membrane depolarization and to activation of voltage-sensitive calcium (Ca^{2+}) channels (Burgoyne 1991). The entry of Ca^{2+} leads to an increase in the concentration of free intracellular Ca^{2+}. The increase of intracellular Ca^{2+} results in the extrusion by exocytosis of the granular contents, including epinephrine, norepinephrine, ATP, some neuroactive peptides or their precursors, chromogranins, and DBH (Winkler et al. 1986). ACh also induces a prompt rise in intracellular cyclic adenosine monophosphate (cAMP), which may be involved in stimulus-secretion coupling (Schneider et al. 1979).

The sympathetic nervous system continuously secretes a small amount of norepinephrine into the blood. It has also been known that adrenergic nerve stimulation induces exocytosis. Stimulation of the sympathetic nervous system results in a large increase in the released amount of norepinephrine. Influx of Ca^{2+} plays an essential role in coupling the nerve impulse, axonal membrane depolarization, and opening of voltage-sensitive Ca^{2+} channels with the release of norepinephrine at adrenergic nerve terminals (Goodman and Gilman 1996). Both L- and N- type Ca^{2+} channels are found on cultured sympathetic neurons (Lipscombe et al. 1988, Thayer et al. 1987).

Recently, there is increasing evidence of the existence of specific proteins localized in sympathetic nerve terminals and chromaffin cells, which are involved in catecholamine release. The cytosolic proteins, protein annexin II, and protein kinase C control Ca^{2+}-dependent exocytosis in adrenal chromaffin cells (Burgoyne et al. 1993, Chasserot-Golaz et al. 1996). Furthermore, it has been indicated that three multimeric complexes of the protein syntaxin, 1) syntaxin and n-sec 1, syntaxin, VAMP (vesicle associated membrane protein), 2) SNAP-25 (synaptosome associated protein of 25 kDa), and 3) syntaxin, VAMP, SNAP-25, alpha SNAP and NSF (N-ethylmaleimide-sensitive fusion protein), are important in this process (Kee et al. 1995). The SNAP proteins also participate in the Ca^{2+}-regulated membrane fusion events that mediated neurotransmitter release (Gutierrez et al. 1995). This result supports the hypothesis that conformational changes in syntaxin, resulting from protein-protein interactions and ATP hydrolysis by NSF, mediate transmitter release.

1.3.4 Breakdown and excretion

The actions of catecholamine are terminated by (1) reuptake into the sympathetic nerve endings; (2) metabolism by the enzymes, catechol-O-methyl transferase (COMT) and monamine oxidase (MAO); (3) deamination; and (4) by renal excretion (Axelrod and Weinshiboum 1972, Kopin 1972, Mass et al.1970). Inactivation of catecholamines occurs mainly through reabsorption into the granules of the sympathetic nerve endings or breakdown. Some of the norepinephrine in sympathetic nerve ending are manufactured there, but some others are also from norepinephrine that has been secreted and then taken up again into the noradrenergic neurons. An active reuptake mechanism is characteristic of noradrenergic neurons. Circulating epinephrine and norepinephrine are also taken up in small amounts by noradrenergic neurons in the autonomic nervous system. In this regard, noradrenergic neurons differ from cholinergic nervous. ACh is not taken up to any appreciable degree, but instead, the choline formed by the action of ACh-esterase is actively taken up and recycled (Goodman and Gilman 1996). Labelled catecholamines are rapidly accumulated in tissues receiving extensive sympathetic innervation and concentrated in sympathetic nerve endings. The tissue uptake such as this disappears after denervation (Brownstein and Hoffman 1994), suggesting the presence of transporters on the plasma membrane of sympathetic nerve fibers (Goodman and Gilman 1996).

The catecholamines are metabolized by the reaction sequences shown in Fig. 1.5, i. e. the metabolism by the enzymes COMT and MAO, followed by excretion via the kidney. MAO is present in most tissues, and the action of MAO on norepinephrine or epinephrine

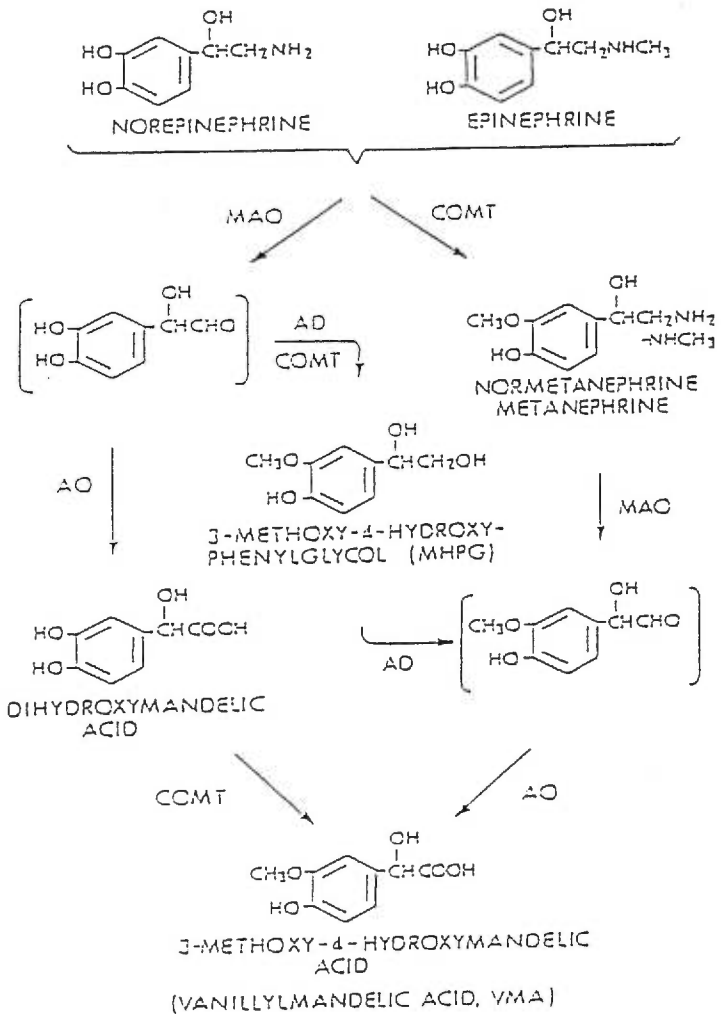

Fig. 1.5 Catecholamine degradation. MAO, monoamine oxidase; COMT, catechol-O-methyl transferase, AD, alcohol dehydrogenase. (From Cryer 1992).

produces the alcohol, 3-methoxy-4-hydroxyphenylglycol (MHPG) or the acid, 3-methoxy-4-dihydroxymandelic acid (DOMA) (Tipton 1973). COMT functions to metabolize circulating catechols in the liver and kidney and to metabolize locally released norepinephrine in the effector tissue. The action of COMT produces normetanephrine from norepinephrine and metanephrine from epinephrine, vanillylmandelic acid (VMA) or MHPG from 3-methoxy-4-hydroxymandelic acid or 3-methoxy-4-hydroxyphenylglycol; 3-methoxytyramine from DA; and homovanillic acid (HVA) from 3-methoxy-4-hydroxyphenylacetic acid (Sharman 1973). Catecholamines and catecholamine metabolites (VMA, MHPG, HVA) are excreted in the urine, a small fraction is excreted unchanged or as O-methylated amines (metanephrines).

1.4. ADRENERGIC RECEPTORS AND ACTIONS OF CATECHOLAMINES

The catecholamines exert their physiologic effects by stimulating specific adrenergic receptors (adrenoceptors) in the plasma membranes of their target cells (Heinsimer and Lefkowitz 1982, Insel 1984). The effects of epinephrine and norepinephrine are brought about by actions on two classes of receptors, α- and β-adrenergic receptors. On the basis of pharmacologic and biochemical studies, α- and β-receptors are further subdivided into α1 and α2 and β1 and β2. A third β-adrenergic receptor (β3) in human gene encoded has been isolated (Emorine et al. 1989, Granneman et al. 1993). β1, β2 and α2 receptors are structurally similar. They are single-unit glycoproteins with molecular weights around 64,000. Alpha-1 receptors differ from the others and the cDNA encoded a single

polypeptide of 515 amino acids with an apparent molecular weights of approximately 80,000 (Cotecchia et al. 1988). The α1 receptors mediate smooth muscle contraction, while α2-adrenoceptors mediate inhibition of various functions.

The experiments in which norepinephrine overflow from perfused cat spleen in response to nerve stimulation before and after treatment with phentolamine or phenoxybenzamine, α–adrenergic antagonists, suggested that the feedback inhibitory effect of norepinephrine on its own release from nerve terminals is mediated by presynaptic α-adrenoceptors (Kirpekar and Puig 1971). Therefore, the presynaptic α–adrenoceptors were designated as α2, whereas the postsynaptic α receptors were designated as α1. The α2- and β2-adrenergic receptors are localized in presynaptic regions and play important roles in the local regulation of neurotransmitter release from sympathetic nerve ending. More recently, however, it has been suggested that α2-adrenoceptors are also present at postjunctional or nonjunctional sites in several tissues, e.g., on many types of neurons in the brain. By contrast, both α1- and β1-adrenergic receptors are located in the vicinity of adrenergic nerve terminals in peripheral target organs, strategically placed to be activated during stimulation of sympathetic nerves. It has been found that α1-adrenoceptors are also functional presynaptically in the autonomic nervous system (Goodman and Gilman 1996).

1.4.1 Alpha-adrenoceptors

The α-adrenergic receptors mediate a variety of response including vasoconstriction.

The translation of α-receptor occupancy into physiological response is mediated by distinct mechanisms for the α1 and α2 receptors.

The α1 receptors are postsynaptic ones (junctional adrenergic receptors) that typically mediate vascular and other smooth muscle contraction. The α1 receptor binds to protein G_q and activates phospholipase C (PLC). This enzyme catalyzes the conversion of phosphatidylinositol (PI) phosphate to inositol triphosphate (IP3) and diacylglycerol. IP3 increases intracellular Ca^{2+} concentration by releasing Ca^{2+} from intracellular stores. Alpha 1 receptor can increase intracellular Ca^{2+} and promote Ca^{2+} influx through voltage-sensitive Ca^{2+} channels to induce physiologic responses (Piascik et al. 1996).

Alpha 2 receptor is coupled to an inhibitory G_i protein, resulting in an inhibition of adenylate cyclase and the subsequent reduction of intracellular cAMP level and protein kinase A activity. Additional signalling pathways have also been demonstrated for the α2-adrenoceptors in expressing transfected α2-adrenoceptors. These signalling pathways include acceleration of Na^+/H^+ exchange (Sweatt et al. 1985), activation of phospholipase A_2 (PLA_2) (Sweatt et al. 1986), activation of phospholipase D (MacNulty et al. 1992) and activation of PLC (Cotecchia et al. 1990). When α2 receptors are activated, the release of norepinephrine is inhibited. The physiological process is currently not clear, although a reduction of Ca^{2+} influx due to the selective inhibition of neuronal N-type Ca^{2+} channels has previously been suggested as an underlying mechanism of presynaptic inhibition of norepinephrine release (Lipscombe et al. 1989).

1.4.2 Beta-adrenoceptors

The β receptor mediates cardiac stimulation, bronchodilation, and vasodilation. Beta 1 and β2 receptors activate adenylyl cyclase via the Gs α subunit to increase intracellular cAMP production, which in turn, as a second messenger, converts protein kinase A to its active form, kinase A, then phosphorylates a variety of proteins including enzymes, ion channels, and receptors (Steer 1977, Pollet and Levey 1980). In addition, Gs can directly enhance the activation of voltage-sensitive Ca^{2+} channels in the plasma membrane of skeletal and cardiac muscle (Brown and Birnbaumer 1988).

1.4.3 Actions of catecholamines

Catecholamines produce a variety of effects in the body, where adrenergic receptors are widely distributed mediating effects of the neurotransmitter of sympathetic nervous system as well as those of circulating catecholamines of adrenal origin. When the body is stressed or in a marked deviation from homeostatic conditions, the sympathoadrenal system is activated resulting in the release of neural noradrenaline and adrenal catecholamines. A summary of physiologic effects of the catecholamines in various tissues is shown in table 1.1.

TABLE 1.1.

Adrenergic responses of selected tissues:

Organ or Tissue	**Receptor**	**Effect**
Heart (myocardium)	β1	Increased force of contraction Increased rate of contraction
Blood vessels	α1 β2	Vasoconstriction Vasodilatation
Kidney	α1 β1	Decreased renin release Increased renin release
Gut	α1, α2, β2 α1	Decreased motility and tone Sphincter contraction
Pancreas	α2 β2	Decreased insulin release, Decreased glucagon release Increased insulin release, Increased glucagon release
Liver	α, β2	Glycogenolysis and gluconeogenesis*
Adipose tissue	β1, (β3)	Increased lipolysis
Skin (apocrine gland on hands, axillae, etc)	α1	Increase sweating
Bronchioles	β2	Dilation
Uterus	α1 β2	Contraction Relaxation

*: There is significant variation among species in the type of receptor that mediates certain metabolic responses; A β3 receptor has been cloned and may mediate responses in fat cells in some species. (From Goldfien 1994).

1.5. REGULATION OF CATECHOLAMINE SECRETION

In the regulation of homeostasis, the autonomic nervous system is responsible for the rapid adjustments of the body to the altered environment. This minute-to-minute regulation is performed at the level of ganglionic synapses and post ganglionic nerve terminals of the autonomic nervous system by the liberation of chemical agents that act transiently on receptors localized at their immediate sites of release. On the other hand, stimulation of the splanchnic nerves to the adrenal medullae causes the release of large quantities of epinephrine and norepinephrine into the systemic circulation, reaching to all tissues of the body. On the average, approximately 80 % of the secreted catecholamines is epinephrine and 20 % is norepinephrine, although the relative proportions can be changed considerably under different physiopathological conditions.

Adrenal medullary catecholamine secretion appears to be regulated by four distinct local mechanisms: (1) cholinergic regulation; (2) adrenoceptor-mediated modulation; (3) L-type Ca^{2+} channel-mediated mechanism; and (4) sensory nerve-mediated peptidergic mechanism (Yamaguchi 1992, Akaike et al. 1990).

1.5.1 Cholinergic regulation of secretion

Cholinergic mechanism of secretion have been studied in primary cultures of bovine adrenal medullary cells (Livett et al. 1983). Both nicotinic and muscarinic receptors participate in the secretion of catecholamines from the adrenal medulla (Akaike et al. 1990).

Nicotinic receptor stimulation causes rapid increase in the permeability to sodium (Na^+) through voltage-sensitive Na^+ channels which brings about an influx of extracellular Ca^{2+}. Thus, an early depolarization appears to serve the principal mechanism for catecholamine release from the chromaffin cells. It has been suggested that nicotinic receptor-mediated catecholamine secretion is exclusively mediated by voltage-dependent entry of extracelluar Ca^{2+}. Protein kinase C (PKC) is likely to serves as a "buffering" second messenger (Tuominen et al. 1992). The short-term activation of PKC is thought to be Ca^{2+} influx (TerBush et al. 1988) whereas that responsibility for long-term activation of PKC is proposed to be nicotine receptor-mediated diacylglycerol formation (Tuominen et al.1992).

The muscarinic receptors also play an important role in secretion of catecholamines from rat chromaffin cells in culture and in the intact gland (Malhotra et al. 1988a, Wakade and Wakade 1983). Muscarinic receptor-mediated release may be caused by Ca^{2+} mobilized from intracellular storage sites (Nakazato et al. 1984, Yamada et al. 1988). By contrast, however, it has been suggested that muscarinic stimulation rises intracellular Ca^{2+} concentration and enhances PI metabolism, not cause the secretion of catecholamines in cultured chromaffin cells (Schneider 1987). The intracellular signalling of muscarinic receptor stimulation thus remains unclear. It is, however, of interest to note that both epinephrine and norepinephrine are non-selectively secreted in response to nicotinic stimulation, whereas epinephrine is selectively released by muscarinic stimulation in the rat adrenal in vitro (Warashina et al. 1989), although the latter observation has not always been confirmed in the canine adrenal in vivo (Yamaguchi 1995).

1.5.2 Adrenoceptor-mediated modulating mechanism

It has been well documented that the release of noradrenaline from sympathetic nerve fibres is modulated by a variety of receptor-coupled mechanisms, particularly presynaptic α2- and β2-adrenoceptor-mediated modulation (Rand et al.1990). Although precise mechanisms regarding either pre- or postsynaptic regulation still remain unclear (Yamaguchi 1992), there exist several indications for adrenoceptor-mediated modulation of adrenal catecholamine secretion. ACh-evoked catecholamine was inhibited by phenylephrine and naphazoline, α-agonists, in bovine adrenal medulla in vitro (Boonyaviroj and Gutman 1979). An α2-agonist, clonidine did not modify adrenal catecholamine secretion induced by direct splanchnic nerve stimulation, but an α2-antagonist, yohimbine, enhanced the secretion. These observation suggest that catecholamine release from the adrenal medulla may be locally modulated by a negative feedback mechanism, mediated through activation of α2-adrenoceptors in vivo (Foucart et al. 1987). It has also been shown that a β2-adrenoceptor agonist enhanced the release of catecholamine induced by ACh from bovine and rat adrenal medullary slices. Adrenal catecholamine response to salbutamol, a β2-adrenoceptor agonist, could also be blocked by either practolol or H 35/25, β2-adrenoceptor antagonists (Boonyaviroj and Gutuman 1977, 1979). A facilitating modulation of adrenal catecholamine release through β2-adrenoceptors localized within the adrenal medulla (either on splanchnic nerve terminals or on chromaffin cells) has been suggested under in vivo conditions (Foucart et al. 1988). Those observations suggest that both α- and

β-adrenoceptors are involved in the local regulation of adrenal medullary secretion, although their functional implication remains inconsistent in different models and approaches (Yamaguchi 1992).

1.5.3 L-type Ca^{2+} channel-mediated mechanism

An increase of intracellular Ca^{2+} concentration is important in regulation of various cellular processes to induce catecholamine secretion in chromaffin cells. Calcium entry into neurons also affects many cellular activities such as neurotransmitter release and gene expression. Alteration of the intracellular Ca^{2+} concentration can be elicited by mobilizing Ca^{2+} from both extra- and intracellular pools (Meldolesi and Pozzan 1987). In adrenal medullary chromaffin cell, the entry of Ca^{2+} is mediated through voltage-dependent Ca^{2+} channels (Montiel et al. 1984) which have been classified into T (transient), L (long lasting), N (neuronal), P (purkinje cells), and Q type (Bean 1989, Hess 1990, Zhang et al. 1993, Tsien et al. 1991).

In vitro studies, the dihydropyridine-sensitive L-type Ca^{2+} channel is blocked by dihydropyridine antagonists (+)-PN 200-110 (Gandia et al. 1987) and activated by Bay-K 8644 (Cena et al. 1989) in chromaffin cell, indicating the presence of dihydropyridine-sensitive L-type Ca^{2+} channels on the surface of chromaffin cell membranes (Motiel et al.1984). It has also been shown that several Ca^{2+} channel blockers, mainly 1,4-dihydropyridine derivatives, depressed potassium- and transmural stimulation-induced catecholamine release from chromaffin cells (Cardenas et al. 1991, Gandia et al. 1987,

Lopez et al. 1989). It has been indicated that L-type Ca^{2+} channels are localized "hot spots" in the plasmalemma of adrenal chromaffin cells where exocytosis occurs (Lopez et al. 1994). These observations suggest that the dihydropyridine-sensitive L-type Ca^{2+} channels are functionally implicated in adrenal catecholamine secretion. Hirning et al. reported that, in adrenal glands, the catecholamine secretion process in response to nicotinic receptor stimulation or to depolarizing stimuli is highly sensitive to dihydropyrdine L-type Ca^{2+} channels, whereas the catecholamine response to splanchnic nerve stimulation is sensitive to N-type Ca^{2+} channels (Hirning et al. 1988). Both L-type dihydropridine-sensitive and dihydropridine-insensitive (non-L type) Ca^{2+} channels might co-operate in controlling exocytosis (Gandia et al. 1995).

Recently, an in vivo study has shown that the dihydropyridine-sensitive L-type Ca^{2+} channels are functionally involved in the regulation of adrenal medullary catecholamine secretion induced by splanchnic nerve stimulation (Gaspo et al. 1993). Furthermore, the dihydropyridine-sensitive L-type Ca^{2+} channels play a role in some peptides such as endothelin-, and angiotensin II-induced adrenal medullary catecholamine secretion in anesthetized dogs (Yamaguchi 1995, Martineau et al. 1996).

1.5.4 Sensory nerve-mediated peptidergic mechanism

Afferent sensory nerves, via the splanchnic nerves, are present in adrenal medulla (Mohamed et al. 1988). It is generally accepted that peptidergic nerves are also present in adrenal. Some peptide immunoreactivities such as for VIP (Hokfelt et al. 1981), substance

P (Kuramoto et al. 1987), NPY (Kuramoto et al.1986), calcitonin gene-related peptide (Kuramoto et al. 1987), corticotrophin-releasing factor (Bruhn et al.1987, Rundle et al. 1988), nitric oxide synthase (Bredt et al. 1990) and PACAP (Frödin et al. 1995) have been localized within nerve fibers innervating the adrenal gland. Moreover, specific immunomodulatory role of the sensory neurons has been suggested.

Substance P-containing sensory neurons are present in the dorsal root ganglia innervating adrenal medulla (Strack et al. 1988, 1989a, b, Zhou et al. 1991). Substance P is released following pretreatment with capsaicin (Bucsics and Lembeck 1981). Substance P has been shown to exert two distinct actions on the nicotinic response to catecholamine secretion of chromaffin cells: (1) Substance P inhibits catecholamine secretory response induced by nicotinic agonists (Livett et al 1979), and (2) Substance P protects against desensitization of nicotinic response (Boksa and Livett 1984, Khalil et al. 1988). In capsaicin-pretreated rats, adrenal catecholamine secretion was reduced in response to insulin, histamine or cold stress (Khalil et al. 1986, 1987). Although the physiological role of sensory afferent in catecholamine secretion has not been fully resolved, the studies suggest that substance P may be the neruomodulator released from such sensory nerves and play a role in modulating cholinergic secretion of catecholamines from the adrenal medulla.

VIP has been shown to be present within chromaffin cells and or nerve fibres in the adrenal medulla of several species (Linnoila et al. 1980). Adrenal catecholamine response to exogenous VIP is mimicked by stimulating the splanchnic nerves (Wakade et al. 1991).

VIP is a noncholinergic transmitter present in adrenal medullary and plays a role for the regulation of catecholamine secretion at low levels of sympathoadrenal activity in the rat adrenal in vitro (Wakade et al. 1991), although the latter concept is not consistently accepted in the adrenal gland in anesthetized dogs (Gaspo et al. 1995). Furthermore, pituitary adenylate cyclase activating polypeptide immunoreactive (PACAP-IR) fibres, the same family of VIP, were observed in the adrenal medulla and are likely to be primarily sensory in origin (Dun et al. 1996, Frödin et al. 1995). PACAP may be involved in the peptidergic sensory nerves which may play a role of regulation of adrenal catecholamine secretion (Dun et al. 1996).

1.6. PITUITARY ADENYLATE CYCLASE ACTIVATING POLYPEPTIDE (PACAP)

ACh is an important secretagogue of catecholamines in the adrenal medulla. However, the splanchnic nerve-stimulated secretion of catecholamines from the adrenal medulla can not be completely blocked by treatment with cholinergic antagonists using either hexamethonium (a selective nicotinic receptor blocker), or pirenzepine (a selective muscarinic receptor antagonist) in anesthetized dogs (Kimura et al. 1992). The release of adrenal catecholamines evoked by exogenous ACh was not blocked by either hexamethonium or atropine; the combination of these drugs produced an inhibition, but the release was not completely abolished (Wakade and Wakade 1983). To explain the remaining catecholamine response, other noncholinergic secretagogues, such as VIP and substance P,

have been suggested to be functionally involved in adrenal catecholamine secretion (Livett and Marley 1993). Furthermore, there is increasing evidence that PACAP is another potent secretagogue of catecholamines in adrenal medulla in various species so far studied (Arimura and Shioda 1995).

1.6.1. Biochemistry of PACAP

PACAP was first isolated from ovine hypothalamus (Miyata et al. 1989). PACAP is a new member of the family of peptides secretin/glucagon/vasoactive intestinal peptide. There exist two isoforms, PACAP38 and PACAP27. The two forms of PACAP result from alternative processing of a precursor protein of 176 amino acids; with 38- and 27-amino acid residues, the latter was derived from the N-terminal 27 of PACAP38. PACAP38 and PACAP27 are equally potent in stimulating pituitary adenylate-cyclase in rat pituitary cell cultures (Miyata et al. 1990). PACAP27 and PACAP38 are also contained in adrenal medulla of several mammalian species. PACAP27 shares 68% homology with VIP and is more potent than VIP in stimulating adenylate cyclase in pituitary cells (Arimura and Shioda 1995) and adrenal chromaffin cell (Watanabe et al. 1992).

1.6.1.1 *Structure of PACAP and VIP*

The amino acid sequence of PACAP has been identified. The structure of PACAP shows considerable similarity to VIP. Comparison of the amino acid sequences of PACAP and VIP is as follows (Arimura and Shioda 1995). Residues that are *underlined* indicate amino acids identical with those for VIP.

PACAP38:

His-Ser-Asp-Gly-Ile-Phe-Thr-Asp-Ser-Tyr-Ser-Arg-Tyr-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Ala-Ala-Val-Leu-Gly-Lys-Arg-Tyr-Lys-Gln-Arg-Val-Lys-Asn-Lys-NH2

PACAP27:

His-Ser-Asp-Gly-Ile-Phe-Thr-Asp-Ser-Tyr-Ser-Arg-Tyr-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Ala-Ala-Val-Leu-NH2

VIP:

His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH2

The molecular cloning of a single hybridizing cDNA for PACAP38 from cDNA libraries of ovine hypothalamus has shown that the nucleotide sequence is different with prepro-VIP (Arimura and Shioda 1995). Ovine pro-PACAP38 contains 176 amino acids where PACAP38 is located between His132 and Lys169 of the precursor (Kimura et al. 1990). PACAP38 and PACAP27 are derived from the same precursor. However, whether PACAP27 is generated from PACAP38 or processed independently from their common precursor remains to be established. The comparison of the sequence of VIP with that of PACAP27 suggests that their great difference in biological activities results principally from the shift from Ala to Gly in position 4 and few substantive differences exist further down the

amino sequences. It is further suggested that the physiologic effects of PACAP and VIP may be mediated by receptor binding with the different spacing of residue pairs (Coy et al. 1996).

1.6.1.2 *Synthesis and distribution*

It is generally accepted that the biosynthesis of biologically active PACAP involves a few of steps from large, inactive precursors to smaller, active products (Mains et al. 1996). PACAP has been suggested to be synthesized in several tissues such as brain and testis (Christophe 1993), but the enzymes involved in this cleavage remain to be elucidated.

RIA binding studies have shown that PACAP binding sites are widely distributed in brain and different peripheral tissues involving testis, adrenal gland, pancreas, lung, liver, stomach, etc (Arimura and Shioda 1995). The distribution of pituitary adenylate cyclase activating polypeptide-like immunoreactive (PACAP-LI) neurons in peripheral tissues is shown in table 1.2. The adrenal gland is one of the peripheral organs which contain a high concentration of PACAP (Arimura et al. 1991). Previous studies have indicated that PACAP like immunoreactivity in all tissues including the adrenal glands in rats is largely PACAP38; PACAP27 being 10% or less of the total PACAP like immunoreactivity (Arimura et al. 1991). More recently, a radioimmunoassay has estimated the quantities of PACAP38 and PACAP27 in the adult rat adrenal gland (table 1.3). The isoform of PACAP found in the adrenal medulla is almost exclusively to be PACAP38 and the quantity of PACAP27 is only 0.25% of that of PACAP38. However, it is still in argument whether PACAP is contained

TABLE 1.2

Distribution of PACAP-LI neurons in peripheral tissues:

Tissue	Cells	Fibres	Species
Respiratory system			
Nose	-	+	Guinea pig, rat
Larynx	-	+	Rat
Trachea	-	+	Rat
Bronchus	-	+	Guinea pig, rat, ferret, pig, sheep, monkey
Lung	+	+	Guinea pig, rat, ferret, pig, sheep, monkey
Digestive system			
Esophagus	+	+	Sheep, cat, human,
Stomach	+	+	Chicken, mouse, rat, sheep, human
Duodenum	+	+	Sheep
Intestine	+	+	Chicken, ferret, cat, pig, sheep. human
Salivary glands	-	+	Rat
Pancreas			
Exocrine	-	+	Mouse, rat, sheep
Endocrine system			
Adrenal cortex			
Adrenal medulla	+b	+e	Mouse, hamster, rat, cow, pig
Pancreas endocrine	+a	+	Mouse, rat, sheep
Urogenital system			
Urinary bladder	-	+	Rat
Reproductive system			
Male	+c	+	Rat, human
Female	-	+	Human
Immune system			
Lymphoid tissue	+d	-	Rat

a: B cell. b: NE cell. c: Germ cell. d: Lymphoid cell (From Arimura and Shioda. 1995).
e: According to new observation, the PACAP-IR fibers were found to contain PACAP38, whereas PACAP27 could not be detected in rat chromaffin cells (From Frödin et al. 1995).

TABLE 1.3

	Medulla (nmol/kg wet tissue)	**Cortex** (nmol/kg wet tissue)
PACAP38	23.9	8.4
PACAP27	0.6	0.3

The concentration of PACAP38 and PACAP27 were determined by radioimmunoassay in extracts of adult rat adrenal gland. From Frödin et al. 1995.

in the adrenal cortex or not. The physiological significance of such differences remains unknown.

1.6.2 PACAP receptors and PACAP receptor antagonists

In mammalian, two classes of PACAP receptors have been described: type I sites, which prefer PACAP over VIP (PACAP-specific site), and type II sites which have equally high affinity for PACAP and VIP (PACAP and VIP shared site). The PACAP type I receptor was found in hypothalamus, brain stem, pituitary, adrenal gland and testes; and further subdivided into PACAP type IA and PACAP type IB receptors. The PACAP type II receptor was expressed in the lung, intestines and liver, while the levels in the brain were five times lower than type I receptor (Ishihara et al. 1992). Previous studies showed that PACAP IA receptor binds PACAP27 with slightly higher affinity than PACAP38 while PACAP IB receptor binds PACAP38 with high affinity and PACAP27 with low affinity (Shivers et al. 1991, Spengler et al. 1993). PACAP type I receptor has been proposed to exist on adrenal chromaffin cells in the rat (Spengler et al. 1993, Moller and Sundler 1996). In contrast, the type II receptor was undetectable in the rat adrenal medulla (Ishiara et al. 1992). The characteristics of the PACAP type I (PACAP-I-R) and type II (PACAP-II-R) receptors are shown in table 1.4.

The PACAP type I receptor virtually do not recognize VIP. However, it has been suggested that PACAP type IA receptors recognize PACAP27 and PACAP38 with equally high affinity and VIP with low affinity. PACAP type IB receptors are present in equivalent

TABLE 1.4

Characteristics of the PACAP type I (PACAP-I-R) and type II (PACAP-II-R) receptors cloned.

EC50 values for stimulation of intracellular cAMP and inositol phosphate production				
	cAMP		Inositol phosphates	
	PACAP38	PACAP27	PACAP38	PACAP27
PACAP-I-R	0.4	0.1	15	>1μM
PACAP-I-R-hip	6	1.7	NS	NS
PACAP-I-R-hop1	0.4	0.1	16	>1μM
PACAP-I-R-hop2	0.4	0.1	15	>1μM
PACAP-I-R-hip-hop1	2.5	0.7	50	>>1μM
PACAP-II-R	0.28	0.42		

PACAP-I-R-hip no stimulating (NS) at all was observed for inositol phosphate production (From Rawlings 1994).

concentrations and recognize PACAP38 with high affinity but PACAP27 with a 100-fold lower affinity (Robberecht et al. 1992). The binding studies have indicated that PACAP38 did not discriminate among the PACAP type IA and IB receptors, whereas PACAP27 could be entirely accounted for by their interaction with PACAP type IA receptors.

It has been suggested that the N-terminally shortened PACAP6-38 and PACAP6-27 fragments acted as competitive antagonists with vastly different potency (*K*i 1.5 nM and 60 nM, respectively) (Vandermeers et al. 1992). In AR 4-2J rat pancreatic acinar cell membranes, PACAP type IA receptors are high affinity (*K*d, 0.3-0.5 nM) for both PACAP27 and PACAP38, and type IB receptors are high affinity for PACAP38 (*K*d, 0.3 nM) but low affinity for PACAP27 (*K*d, 20 nM) (Robberecht et al. 1992). The inhibitory effects of PACAP6-27 preferably with their ability to occupy PACAP type IA receptors (Robberecht et al 1991, 1992). Recent research has shown that both PACAP6-27 and PACAP6-38 blocked the PACAP27, PACAP38 and VIP-stimulated cAMP accumulation in the osteoblast-like tumour cell line (Kovacs et al. 1996). However, VIP antagonist did not reduce the PACAP- or VIP-stimulated cAMP accumulation (Kovacs et al. 1996). Furthermore, VIP-induced adrenal catecholamine release was significantly inhibited by PACAP6-27 while VIP antagonist did not inhibited the VIP-induced catecholamine secretion in anesthetized dogs (Gaspo et al. 1997). Taken together, it can be speculated that PACAP6-27 and PACAP6-38 are potent PACAP receptor antagonist.

1.6.3 PACAP immunoreactivity in the adrenal

It has been reported that the splanchnic nerve terminals were negative in PACAP immunoreactivity in the rat adrenal gland (Shiotani et al. 1995). However, PACAP-IR nerve fibres were indeed found in the rat adrenal gland (Frödin et al. 1995, Dun et al. 1996), and these fibres are likely to be sensory in nature (Dun et al. 1996). This is also supported by the observation that the treatment of rats with capsaicin resulted in a nearly complete disappearance of PACAP-IR fibres in the adrenal gland (Dun et al. 1996). A high concentration of PACAP has been detected by radioimmunoassay in rat adrenal gland (Frödin et al. 1995). The endogenous PACAP is released upon splanchnic nerve stimulation in isolated rat adrenal gland (Wakade et al. 1992). These observations raise the possibility that PACAP may be involved in the peptidergic sensory nerves which may play a role of local regulation of catecholamine secretion in conjunction with cholinergic sympathetic preganglionic fibers (Dun et al. 1996).

PACAP-positive cells were immunoreactive to tyrosine hydroxylase and dopamine β-hydroxylase, but not to phenylethanolamine-N-methyl transferase, suggesting that they were coincident with norepinephrine secreting cells (Shiotani et al. 1995). This observation suggested that PACAP immunoreactivity exist in some cell groups in the adrenal medulla, whereas it can not be observed in the adrenal cortex. In agreement with these observations, immunohistochemical studies have revealed that, in all species so far examined, adrenal PACAP27-LI was confined to the norepinephrine containing cells (Tabarin et al. 1994). Furthermore, PACAP38-LI also localized in norepinephrine containing cells in the adrenal medulla in rats (Shiotani et al. 1995). The PACAP immunoreactivity detected in the

adrenomedullary cells may result either from the local synthesis of PACAP or the uptake of PACAP by splanchnic nerve fibres (Tabarin et al. 1995). However, it is unclear why PACAP is localized only in norepinephrine containing cells. The physiological role of PACAP in norepinephrine storing cells also remains obscure.

1.6.4 Physiological effects of PACAP

In peripheral organs, the effect of PACAP are likely to be mediated either through type I or type II receptor. Various effects so far observed in peripheral organs are summarized in table 1.5. PACAP stimulates insulin release in isolated perfused rat pancreas (Kawai et al. 1992) and augments basal and carbachol-stimulated glucagon secretion as well as plasma glucose associated with a modest elevation of plasma insulin levels in vivo in mice (Fridolf et al. 1992). It has been suggested that PACAP may act as a physiological regulator of the islet hormone release and thereby of the glucose metabolism (Yada et al. 1996). PACAP is involved in the memory storage in drosophila (Kandel and Abel 1995). PACAP prevents neuronal cell death and functions as a neurotrophic factor (Arimura et al. 1994). In the heart, PACAP exerts a long-lasting tachycardia (Sawangjaroen et al. 1992, Will-Shabab et al. 1993), and a potent positive inotropic effect (Minkes et al. 1992). PACAP relaxes intestinal smooth muscle (Schwörer et al 1992).

PACAP is a potent secretagogue in chromaffin cells (Watanabe et al. 1992, Isobe et al. 1993), in isolated perfused adrenal gland (Chowdhury et al. 1994) and in anesthetized rats (Watanabe et al. 1995). Adrenal gland is one of organs which contains highest

TABLE 1.5

Effect of PACAP in peripheral organs.

Organ or Tissue	**Receptor**	**Effect (in vitro)**	**Effect (in vivo)**
Heart (myocardium)	Type I, Type II and PACAPR-3	Inotropic (+) Increased heart rate Seebeck et al. 1996, Will-Shabab et al. 1993, Minkes et al. 1992	Inotropic (+) Chronotropic (+) Increased heart rate Runcie et al. 1995, Suzuki et al. 1993
Blood vessels	Type II	Vasodilator Champion et al. 1996	Vasodilation Warren et al. 1992
Bronchioles	Type I	Bronchodiators Cardell et al. 1991	Bronchodiators Linden et al. 1995
Pancreas	Type I	Insulin increase Kawai et al. 1992	Insulin increase Glucagon increase Yamaguchi. 1996, Fridolf et al. 1992
Liver	Type II	Increased glucose output Yokota et al. 1995	Increased glucose output Trabelsi and Yamaguchi. 1997.
Gastrointestinal	Type I	Relax intestinal smooth muscle Katsoulis et al. 1993, Mungan et al. 1992	Relaxation of intestinal smooth muscle Schwôrer et al. 1993
Adrenal medulla	Type I	Increased CA release Isobe et al. 1993	Increased CA release Watanabe et al. 1995

PACAP receptors mediate certain physiology responses in peripheral organs. A PACAPR-3 receptor has been cloned and may mediate responses in heart in some species. Type I and Type II: indicate PACAP type I and type II receptors, respectively.

concentration of PACAP (Arimura et al. 1991). PACAP type I receptor has been detected in rat adrenal medulla. PACAP exhibits a variety of biological activities, including activation of adenylate cyclase in PC-12 cells and other cells (Watanabe et al. 1990, Miyata et al. 1989); stimulating cAMP production in porcine adrenal medulla chromaffin cells (Isobe et al. 1993, Watanabe et al. 1992); increase in intracellular Ca^{2+} concentrations in bovine adrenal medulla (Tanaka et al. 1996). More recently, it has been suggested that PACAP-induced catecholamine secretion is involved in PACAP receptor-mediated intracellular cAMP increase and Ca^{2+} influx in rat adrenal chromaffin cells (Przywara et al. 1996). However, all these mechanisms shown in *in vivo* studies still remain unproven under in vivo conditions.

1.7. HYPOTHESES AND OBJECTIVES

1.7.1 Hypotheses

It has been shown that in cultured chromaffin cells, the Ca^{2+} influx into the chromaffin cells and resultant increases in intracellular Ca^{2+} concentration are essential for nicotine-evoked catecholamine release (Garcia et al. 1984). It has further been demonstrated that the dihydropyridine-sensitive L-type Ca^{2+} channels, presumably localized on the surface of the chromaffin cell membranes are functionally involved in adrenal catecholamine secretion induced by nicotinic stimulation both in vitro (Gandia et al. 1987, Garrido et al. 1991, Montiel et al. 1984) and in vivo (Gaspo et al. 1994). In more recent in vivo studies, the functional implication of the dihydropyridine-sensitive L-type Ca^{2+} channels has been

indicated in adrenal catecholamine secretory response to non-cholinergic stimulation by certain peptides such as endothelin-1 (Yamaguchi 1993, 1995) and angiotensin-II (Martineau et al. 1996). These in vivo observations are consistent with those obtained from various in vitro studies (Boarder and Marriott 1991, Stauderman and Pruss 1989). Thus, the dihydropyridine-sensitive L-type Ca^{2+} channels are also likely to be a common factor functionally involved in noncholinergic peptidergic stimulation-induced adrenal catecholamine secretion.

Nevertheless, it appears unlikely that the dihydropyridine-sensitive L-type Ca^{2+} channels play a functional role in catecholamine release induced by VIP, a noncholinergic neurotransmitter in rat adrenal gland (Wakade et al. 1991). In support of this hypothesis, a recent in vivo study has indicated that the VIP-induced increase in adrenal catecholamine secretion was not significantly inhibited in the presence of nifedipine (Gaspo et al. 1997). This observation is compatible with a previous finding that VIP-induced catecholamine secretion was not rapidly lost in Ca^{2+}-free medium (Marley 1987). More recently, PACAP was discovered and shown to be a potent inducer of catecholamine secretion in adrenal medulla. However, catecholamine release induced by PACAP, a new member of the VIP / glucagon / secretin family, is likely to result from an increase in Ca^{2+} influx by selectively activating voltage-dependent L-type Ca^{2+} channels in cultured porcine chromaffin cells (Isobe et al. 1993, Tanaka et al. 1996). Interestingly, however, nifedipine had no effect on PACAP-induced catecholamine secretion in cultured rat chromaffin cells (Przywara et al. 1996). It seems, therefore, likely that the functional implication of the dihydropyridine-

sensitive L-type Ca^{2+} channels in PACAP-induced catecholamine secretion appears to be specific depending on the species used. Furthermore, the potential implication of L-type Ca^{2+} channels in PACAP-induced catecholamine release remains unproven under in vivo conditions. Therefore, the hypotheses to be tested in the present study is that the L-type Ca^{2+} channels are involved in PACAP-induced catecholamine release in the canine adrenal gland in vivo.

1.7.2 Specific aims

The principal objective of the present study was to investigate local effect of PACAP on the canine adrenal medulla, and to clarify whether the dihydropyridine-sensitive L-type Ca^{2+} channels are involved in PACAP-induced adrenal catecholamine secretion in the dog model in vivo.

The specific aims were:

1) to confirm if the catecholamine response to PACAP is dose-dependent;

2) to compare the catecholamine secretory effect of PACAP27with that of PACAP38;

3) to demonstrate the anticipated catecholamine response to PACAP is due to its direct action on the adrenal medulla; and

4) to indicate that the dihydropyridine-sensitive L-type Ca^{2+} channels are functionally

involved in PACAP-induced catecholamine release under in vivo conditions.

CHAPTER 2

A STUDY ON THE ROLE OF L-TYPE Ca^{2+} CHANNEL IN PACAP-INDUCED ADRENAL CATECHOLAMINE RELEASE IN VIVO*

* This work has recently been published in the American Journal of Physiology as follows: **Geng, G.**, R. Gaspo, F. Trabelsi, and N. Yamaguchi. Role of L-type Ca^{2+} channel in PACAP-induced adrenal catecholamine release in vivo. American Journal of Physiology 273 (Regulatory Integrative Comp. Physiol. 42) R1339-R1345, 1997.

ABSTRACT

The aim of the present study was to investigate whether the dihydropyridine-sensitive L-type Ca^{2+} channel is operative in adrenal catecholamine (CA) secretion induced by a novel neuropeptide, pituitary adenylate cyclase activating polypeptide (PACAP), in anesthetized dogs. Plasma CA concentrations in adrenal venous and aortic blood were determined by an HPLC method. All drugs tested were locally infused to the left adrenal gland via the left adrenolumbar artery. PACAP, with the isoform consisting of 27 (PACAP27) and 38 (PACAP38) amino acid residues, significantly increased CA output in a dose-dependent manner with doses ranging from 5 to 500 ng and 7 to 700 ng, respectively. However, the amplitude of epinephrine response to PACAP27 was 3 times greater than that obtained with PACAP38 at the highest dose tested. In a separate group, a single dose of PACAP27 (50 ng) induced highly reproducible CA responses when the same dose was repeated with an interval of 35 min. In dogs treated with nifedipine (50 μg), 5 min prior to the second administration of PACAP27, the net CA response was significantly inhibited by ~ 50% as compared to that obtained in the presence of vehicle. A similar CA response to BAY-K 8644 (5 μg) was completely abolished by the same dose of nifedipine. The present results indicate that both PACAP27 and PACAP38 have the direct local secretagogue effect on the adrenal medulla in vivo, and that CA responses to PACAP27 were greater than those observed with PACAP38 at equivalent mole doses. The study suggests that the dihydropyridine-sensitive L-type Ca^{2+} channel is functionally involved in PACAP-induced adrenal CA secretion in the canine adrenal medulla in vivo.

Key words: PACAP27, PACAP38, BAY-K 8644, nifedipine, medullary secretion, dogs

INTRODUCTION

Pituitary adenylate cyclase activating polypeptide (PACAP) is a novel member of the family of vasoactive intestinal polypeptide (VIP). This biologically active neuropeptide exists in two forms, PACAP38 and PACAP27 with 38 amino acid residues and the N-terminal amidated 27 residues, respectively, originally isolated from ovine hypothalamus (16, 18). The structure of PACAP27 has been shown to be 68% homologous to VIP in their amino acid sequences (10, 18). PACAP is widely distributed in central nervous system and peripheral organs. The adrenal gland is the one which contains a high concentration of PACAP (1). PACAP is a very potent secretagogue for catecholamines in cultured chromaffin cells from the rat and pig as well as in the isolated, perfused rat adrenal gland (10, 13, 25). PACAP has been postulated to play a role of non-cholinergic neurotransmitter in the rat adrenal medulla (24, 26), but its local direct effect on the adrenal gland has not convincingly been demonstrated in other in vivo models. On the other hand, it has been shown that, in cultured porcine chromaffin cells, PACAP-induced catecholamine release was inhibited by L-type Ca^{2+} channel blockers (13), suggesting that PACAP increases Ca^{2+} influx into chromaffin cells and thereby induces the release of catecholamines. Furthermore, it has been demonstrated that the release of adrenal catecholamine depends on the influx of Ca^{2+} into the intracellular compartment of chromaffin cells through voltage-dependent Ca^{2+} channel (4, 15). The dihydropyridine-sensitive L-type Ca^{2+} channel has thus been locally implicated in adrenal catecholamine secretion induced by angiotensin II and endothelin-1 under in vivo conditions (17, 28). However, the potential involvement of L-type Ca^{2+} channel in PACAP-induced catecholamine release still remains unknown in vivo. Therefore,

the specific aims of the present study were to investigate whether PACAP possesses the direct secretagogue effect in vivo, and the dihydropridine-sensitive L-type Ca^{2+} channel is functionally involved in PACAP-induced catecholamine secretion in the canine adrenal gland under in vivo conditions.

METHODS

General preparation of animals. Adult mongrel dogs (27.0 ± 0.8 kg, n = 39), fasted overnight but allowed free access to water, were anesthetized with pentobarbital sodium (30 mg/kg iv, followed by 4 mg/kg as needed (MTC pharmaceuticals, Cambridge, Ontario). Respiration was controlled through an endotracheal tube, with room air delivered by a respirator (model 607; Harvard, South Natick, Mass). Body temperature of each dog was monitored and kept constant at 37.5 ± 0.5°C by a thermoregulator (model 74; Yellow Spring Instruments Yellow springs, Ohio) connected to a heating pad throughout the experiment. Physiological saline was slowly administered intravenously during the whole period of the experiment to prevent dehydration. The pH of physiological saline was adjusted between 7.35-7.45 immediately before use. Both femoral arteries were cannulated: the right femoral artery was used to measure aortic pressure through a catheter, the tip of which was placed at the level of abdominal aorta, and the left femoral artery to obtain aortic blood samples.

Preparation of local intra-arterial drug infusion into left adrenal gland. The experimental model used in this study has been reported elsewhere in full detail (27). Briefly,

after a median laparotomy and a left flank incision, the left adrenolumbar artery was dissected free from the surrounding tissues and cannulated in a retrograde manner, so that the tip of catheter (PE-90) was placed either close to or underneath the gland. The volume of this catheter was fixed at 0.5 ml. All visible branches arising from the adrenolumbar artery toward the outside of the gland were ligated to prevent undesired drug diffusion into the systemic circulation. The catheter was connected to an infusion pump (model 1140-001, Harvard, South Natick, Mass).

Preparation of extracorporeal adrenal venous circuit. A specially shaped catheter (PE-240) was inserted into the adrenal vein through the left femoral vein. The volume of this catheter was fixed to be 1.5 ml. The catheter was tied at the adreno-abdominal vena caval junction to prevent dilution of adrenal venous blood with abdominal vena caval blood. The left adrenolumbar vein distal to the gland was ligated to obtain actual adrenal venous blood. Adrenal venous blood from the left gland was drained into a small reservoir. The blood in the reservoir was connected to a perfusion pump (Masterflex 7016-52, Cole -Parmer Instrument, Chicago, IL) to return adrenal venous blood through a catheter inserted into the right femoral vein at a rate of stabilized initial venous blood flow (27). After all surgical procedures were completed, heparin sodium (200 U/kg iv) was administered, followed by 100 U/kg every hour thereafter. The dog was then allowed a stabilization period of ~ 60 min.

Measured parameters. Aortic blood pressure and heart rate measured from the body

surface ECG were monitored with a polygraph system (model RM-6000, Nihon-Kohden, Tokyo, Japan). Blood samples for plasma catecholamine assays were collected in ice-cooled test tubes. Left adrenal venous blood flow was determined by a gravimetric method, and haematocrit with microhematocrit capillary tubes after 10 min centrifugation. An aliquot of 1.5 ml of aortic and adrenal venous blood were transferred to a chilled centrifuge tubes containing 30 µl of preservative solution (pH 6.5) consisted of EGTA (95 mg/ml) and glutathione (60 mg/ml). Blood samples for catecholamine measurements were immediately centrifuged at 4°C for 5 min at 15,800 g (Centrifuge Eppendorf, model 5402, Eppendorf, Hamburg, Germany). Plasma was stored at -80°C until the assay carried out within two weeks. Plasma epinephrine and norepinephrine concentrations were determined by an electrochemical detector (model 5200, ESA Coulochem II Multi-Electrode Detector, Bedford, MA) coupled with a high performance liquid chromatographic system (Gilson, Villiero-le-Bel, France) according to the method previously reported from our laboratory (27). At the end of each experiment, the left adrenal gland was removed and weighed. The net catecholamine output was calculated as follows: Net output of adrenal catecholamines ($ng \cdot min^{-1} \cdot g^{-1}$) = $([CA]_{ADV}-[CA]_{AO}) \times BF_{ADV} \times (1-Ht_{ADV})$/wet weight of adrenal gland, where $[CA]_{ADV}$ is plasma catecholamine concentration in adrenal venous blood, $[CA]_{AO}$ is circulating catecholamine concentration in aortic blood, BF_{ADV} is adrenal venous blood flow, and Ht_{ADV} is adrenal venous hematocrit. The amount of catecholamine released during the first 5 min after PACAP27 was obtained from an area under the curve of net catecholamine output, and the data were expressed as $ng \cdot g^{-1}$ of wet weight of the gland.

Experimental groups and drugs. The present study was carried out in five separate groups: the first two groups served to compare local effects of PACAP27 (M.W. = 3147.6) (n = 11) and PACAP38 (M.W. = 4534.3) (n = 7) (Sigma Chemical, St-Louis, MO) on the adrenal medulla within a dose range from 5 to 500 ng or 7 to 700 ng, respectively; the third group (n = 8), serving as the control group, was to ensure the reproducibility of the adrenal catecholamine responses to repeated single doses of PACAP27 (50 ng) in the presence of vehicle (6% ethanol prepared with saline); the fourth (n = 9) and fifth (n = 4) group served to test the effect of nifedipine (50 μg) (Sigma Chemical) on the adrenal catecholamine response induced by PACAP27 (50 ng) and BAY-K 8644 (5 μg) (Calbiochem La Jolla, CA), respectively. The doses of nifedipine and BAY-K 8644 were selected based on our recent observations that nifedipine at the dose used in the present study completely blocked the adrenal catecholamine response to BAY-K 8644 in a similar experimental setup (17). The dose of PACAP27 was selected according to the present dose-response experiments. PACAP27 was dissolved in saline. Nifedipine and BAY-K 8644 were first dissolved in ethanol and then diluted with saline to the desired concentration; the final solution contained 6% ethanol.

Experimental protocols. In the first two separate groups, in which the dose-dependency was tested, dogs received, with an interval of 30 min, three different doses of either PACAP27 (5, 50 and 500 ng) or PACAP38 (7, 70 and 700 ng) with the same molar basis (3.2, 32 and 320 nM) for both drugs. One minute after the initial control sample was taken, the first dose of either drug was infused into the left adrenolumbar artery at a rate of

0.5 ml/min. The net infusion period was fixed to be 1 min. The dead volume of the adrenal arterial (0.5 ml) and venous (1.5 ml) catheter was taken into account in relation to the infusion rate and adrenal venous blood flow, respectively. Blood collections were made at 1, 2, 3, 5, 10, 15 and 30 min after the onset of net infusion. The sample obtained 30 min after the drug infusion served as control for the next response. The procedure was repeated in a smilar way for the next two higher doses. Two additional samples were collected 105 and 120 min after the third dose to ensure the stability of basal catecholamine secretion.

In the third group receiving the vehicle and PACAP27, immediately after taking the initial control sample, the vehicle was infused into the left adrenolumbar artery in a similar way as described above. Then, the second control sample was taken 5 min after the vehicle administration. PACAP27 (50 ng) was similarly administered, and blood samples were collected at 1, 2, 3, 5, 10, 15 and 30 min after the infusion of PACAP27. As in the case of the first group, the sample obtained 30 min after PACAP27 served as control for the next procedure. Then, the second vehicle was administered followed by the same procedures as those after the first vehicle administration.

In the fourth and fifth group, the effect of PACAP27 (50 ng) and BAY-K 8644 (5 μg) was tested in the presence of nifedipine (50 μg), respectively. The experimental protocol was exactly the same as that described for the third group with the exception that the second vehicle was replaced with nifedipine administration.

All surgeries and experimental procedures were carried out under the full surgical anesthesia. All experiments were acute, terminal procedures. At the end of the experimental protocols, each animal used in this study was euthanized following an intravenous overdose of pentobabital sodium without regaining consciousness.

Statistical analyses. The statistical evaluations and the calculations of an area under the curve were carried out using a statistical software package (SigmaStat and SigmaPlot for Windows, Version 1.0, Jandel Scientific, San Rafael, CA). Differences within subjects observed over a given experimental period were evaluated by an analysis of variance for repeated measures followed by multiple comparisons with one control using the Dunnett's method (Figs.1, 3, 5 and 6). Comparisons of catecholamine responses to PACAP27 before and after nifedipine administration were made using the paired t-test (Fig. 4). Comparisons between different groups were conducted by the use of one-way analysis of variance followed by the Bonferroni's test (Figs. 2 and 7). The results are expressed as mean ± SE, and $P < 0.05$ was considered statistically significant.

RESULTS

Effect of PACAP27 and PACAP38 on adrenal catecholamine output. Following local infusion of either PACAP27 or PACAP38, both adrenal venous catecholamine concentration and blood flow increased without significantly affecting circulating catecholamine concentration in aortic blood. Consequently, the net output of adrenal epinephrine and norepinephrine significantly increased in a dose-dependent manner in

response to various doses of PACAP27 ranging from 5 to 500 ng (Fig.1). The response was rapid, and the peak response was usually observed during the infusion (Fig. 1). After the cessation of the infusion, the increased catecholamine output returned towards the corresponding pre-infusion control levels within ~ 5, ~ 10 and ~ 15 min following the dose of 5, 50 and 500 ng, respectively (Fig. 1). Thus, the duration of action also increased in a dose-dependent manner. Similar dose-related responses were observed with PACAP38, but the maximum responses were significantly smaller than, being ~ 1/4 to ~ 1/3 of, those obtained with PACAP27 (Fig. 2). Mean aortic pressure and heart rate did not significantly change following the local administration of either peptide at any doses tested.

Catecholamine responses to PACAP27 in the absence or presence of nifedipine. In the control group receiving vehicle, adrenal catecholamine output significantly increased in response to PACAP27 with a dose of 50 ng. The increases in the output of both epinephrine and norepinephrine were highly reproducible upon the second administration of PACAP27 with the same dose given at an interval of 35 min (Fig. 3). The increased catecholamine output remained significantly higher than the control levels up to at least 5 min after the administration of PACAP27 (Fig. 3). The quantity of each epinephrine and norepinephrine released during the first 5 min after the onset of PACAP27 infusion remained unchanged upon the second infusion in the vehicle control group (Fig. 4A).

In the group receiving nifedipine (50 μg), the maximum catecholamine response to PACAP27 observed in the presence of nifedipine was slightly attenuated by ~ 30% (P =

0.02 and 0.16 for epinephrine and norepinephrine, respectively) as compared with the control response (Fig. 5). In addition, the catecholamine output remained significantly elevated only for 2 min in the presence of nifedipine (Fig. 5). Consequently, the quantity of catecholamines released during the first 5 min after the administration of PACAP27 was significantly reduced by ~ 50% in the presence of nifedipine (Fig. 4B). This reduction in PACAP27-induced catecholamine secretion was significantly different from the value observed in the vehicle control group (Fig. 7). By contrast, the increase in catecholamine output similarly observed in response to BAY-K 8644 was almost completely blocked by nifedipine (Figs. 6 and 7).

DISCUSSION

The present study demonstrates that the local administration of either PACAP27 or PACAP38 into the left adrenolumbar artery resulted in a significant increase in adrenal catecholamine secretion in a dose-dependent manner without any significant systemic effect in anesthetized dogs. The study also shows that the amplitude of catecholamine response to PACAP27 was at least 3 times greater than that to PACAP38 at the highest dose tested in the dog model in vivo. The results suggests that the dihydropyridine-sensitive L-type Ca^{2+} channel is functionally involved in mechanisms regulating adrenal catecholamine secretion induced by PACAP27.

Since PACAP has been originally isolated from ovine hypothalamic tissues, it has been suggested that PACAP may play distinct roles as a neurotransmitter, neuromodulator,

or neurotropic factor in the central nervous system (2). In the peripheral nervous system, PACAP has been proposed to be a non-cholinergic neurotransmitter controlling catecholamine secretion in the rat adrenal medulla (24). PACAP has been shown to be a potent secretagogue in various studies in vitro, including the isolated, perfused rat adrenal gland (11) and cultured chromaffin cells obtained from rat (25), frog (29) and porcine adrenal glands (13). More recently, it has been shown that PACAP increased catecholamine secretion from the rat adrenal in vivo (26). In concordance with those previous studies, the present observations are compatible with the view that PACAP may be functionally involved in local regulation of adrenal catecholamine secretion as a potent modulator or secretagogue in anesthetized dogs. Furthermore, we observed, in the present study, that mean aortic pressure, heart rate and circulating catecholamine levels in aortic blood did not significantly change even during the infusion of either PACAP27 or PACAP38 at their highest dose tested. In this context, it is of interest to note that, in anesthetized dogs, intravenous injection of PACAP27 resulted in either hypotension or hypertension associated with tachycardia or bradycardia, respectively, depending on the doses administered (12). The latter observations suggest that PACAP may affect the sympathoadrenal activity through cardiovascular reflexes. However, the results of the present study are consistent with the view that the increasing effect of PACAP on catecholamine secretion resulted from its direct action on the adrenal medulla and not from secondary factors such as a reflex-induced increase in sympathetic outflow.

Although adrenal chromaffin cells possess not only L-type but also N-, P-, and Q-

type voltage-dependant Ca^{2+} channels (7), it is well accepted that L-type Ca^{2+} channel is a major route of Ca^{2+} entry (15). In the present study, BAY-K 8644, a dihydropyridine L-type Ca^{2+} channel activator, significantly increased catecholamine secretion. This BAY-K 8644-induced catecholamine release was almost completely diminished by nifedipine, clearly indicating the specific antagonism at the level of the dihydropyridine-sensitive L-type Ca^{2+} channel. With respect to the functional existence of L-type Ca^{2+} channel in the dog adrenal medulla in vivo, we have recently demonstrated that the increase in adrenal catecholamine output induced by BAY-K 8644 was significantly inhibited in the presence of nifedipine in a dose-dependent manner under conditions similar to those of the present study (17). These observations are compatible with the view that the dihydropyridine-sensitive L-type Ca^{2+} channel is operative in the local regulation of adrenal catecholamine secretion not only in vitro (4) but also under in vivo conditions (8, 17, 28).

It has been debatable whether the dihydropyridine-sensitive L-type Ca^{2+} channel is functionally involved in PACAP-induced adrenal catecholamine secretion. It has been shown that, in cultured porcine chromaffin cells, catecholamine release induced by PACAP resulted, most probably, from an increase in Ca^{2+} influx by selectively activating voltage-dependent L-type Ca^{2+} channel, but not N-, P-, or Q-type channels (13). More recently, the whole cell patch-clamp technique revealed that, in bovine adrenal chromaffin cells, PACAP causes both Ca^{2+} release, mainly from caffeine-sensitive Ca^{2+} stores, and Ca^{2+} influx through dihydropyridine-sensitive L-type Ca^{2+} channel (23). In cultured rat chromaffin cells, however, nifedipine had no effect on PACAP-induced catecholamine secretion (20). These

previous in vitro studies imply the existence of species difference with respect to mechanisms involved in PACAP-induced adrenal catecholamine secretion.

In the present study, the maximum increase in catecholamine response to PACAP27, usually observed during the first minute of infusion, was inhibited by ~ 30% in the presence of nifedipine, while the quantity of catecholamines released during the first 5 min was diminished by ~ 50%. The lack of more complete inhibition of the PACAP27-induced response, particularly of the initial rapid increase in catecholamine output, may be due to the dose of nifedipine which was insufficient. This is, however, unlikely to be the case, because the similar catecholamine response to BAY-K 8644 was abolished in the presence of nifedipine with the same dose used for the experiments with PACAP27 in the present as well as in our previous study (17). Furthermore, we have shown that a small dose of nifedipine (1/10 of that used in the present study) markedly attenuated by ~ 75% the initial steep catecholamine response to endothelin-1, an extent of which was similar to that induced by PACAP27, in the same dog model used in the present study (28). In addition, the rapid increase in catecholamine response to angiotensin II was also significantly inhibited by ~ 65% in the presence of nifedipine under conditions similar to those of the present study (17). Taken together, the present findings are compatible with the hypothesis that the Ca^{2+} entry via the dihydropyridine-sensitive L-type Ca^{2+} channel may be involved, but play only a minor role, to initiate the exocytotic release of catecholamines induced by PACAP27 in the canine adrenal medulla in vivo.

It may be of further interest that the PACAP27-induced increase in catecholamine output remained significantly elevated at least for 5 min, while it remained only for 2 min in the presence of nifedipine. It is, therefore, likely that the duration of action of PACAP27 was significantly shortened by nifedipine. This observation also suggests that the dihydropyridine-sensitive L-type Ca^{2+} channel is functionally involved in PACAP27-induced adrenal catecholamine secretion, but its contribution to the initial rapid Ca^{2+} entry may not be as important as observed in catecholamine release induced by either endothelin-1 (28) or angiotensin II (17). In this context, we have recently observed that VIP-induced adrenal catecholamine secretion remained unchanged in the presence of nifedipine at the same dose used in the present study (9). The latter finding is compatible with the view that the dihydropyridine-sensitive L-type Ca^{2+} channel may play only a minor role in PACAP27-induced catecholamine secretion, as PACAP27 is 68% homologous to VIP in their amino acid sequences and, therefore, shares mechanisms mediated by cyclic adenosine monophosphate (AMP) (10, 18). Indeed, it has been postulated that, in rat chromaffin cells, PACAP-induced catecholamine release could be initiated by a nifedipine-resistant, cyclic AMP-mediated Ca^{2+} influx (20).

In conclusion, PACAP is a potent adrenomedullary secretagogue in anesthetized dogs. Both PACAP27 and PACAP38 directly stimulate the medulla resulting in an increase in catecholamine release in a dose-dependent manner. It is most likely that the dihydropyridine-sensitive L-type Ca^{2+} channel is functionally involved in mechanisms controlling catecholamine secretion induced by PACAP27.

PERSPECTIVES

It has been shown that intravenous administration of PACAP produced an increase in systemic blood pressure which was blocked by an α-adrenoceptor antagonist, phentolamine, in anesthetized dogs (21) and cats (5) as well as in adrenalectomized cats (5). Furthermore, PACAP has been shown to exert a central pressor action by increasing sympathetic outflow in anesthetized dogs (22). These observations strongly suggest that PACAP may play important roles in hormonal and neural control of cardiovascular functions. On the other hand, nifedipine and other similar L-type Ca^{2+} channel blockers are commonly used in the treatment of cardiovascular diseases such as hypertension and cardiac arrhythmias, which may frequently involve sympathoadrenal dysfunctions (6, 19). In this context, the present findings may contribute to a better comprehension of mechanisms of action of PACAP and its interaction with the L-type Ca^{2+} channel in the sympathoadrenal system under certain pathophysiological conditions.

With respect to the potential interaction of PACAP with the peripheral autonomic nervous system, it has been shown that PACAP elicited sustained release of catecholamines in cultured superior cervical ganglion (3). In cultured porcine adrenal medullary chromaffin cells, PACAP has also been shown to stimulate sustained catecholamine production resulting from both cyclic AMP- and protein kinase C-dependent activations of tyrosine hydroxylase and dopamine β-hydroxylase (14). Furthermore, PACAP27 potentiated the cardiac slowing induced by vagal stimulation, while had no effect on the cardiac response to sympathetic stimulation in anesthetized dogs (21). PACAP38 has similarly been shown

to activate cardiac parasympathetic nerves in the isolated, blood-perfused dog heart (30). These observations suggest that PACAP may facilitate cholinergic, rather than adrenergic, neuroeffector transmission. Our recent study indicated that splanchnic nerve stimulation-induced catecholamine secretion was significantly enhanced in the presence of PACAP27 in the canine adrenal gland in vivo (unpublished observation). The latter observation is compatible with the view that PACAP facilitates the cholinergic neurotransmission in the adrenal medulla. However, the functional implication of either presynaptic receptor-mediated or nicotinic and/or muscarinic postsynaptic receptor-mediated mechanisms remains to be elucidated.

ACKNOWLEDGMENTS

This work was supported by grants from the Canadian Diabete Association and, in part, from the Heart and Stroke Foundation of Québec as well as from the Medical Research Council of Canada. G. Geng is a holder of a studentship from the Groupe de recherche sur le système nerveux autonome (GRSNA) of the Université de Montréal. During this study, R. Gaspo was a holder of Studentship supported by the Medical Research Council of Canada. The authors wish to thank Sanae Yamaguchi and Richard Lalande for their skilled and devoted technical assistance.

REFERENCES

1. **Arimura A., A. Somogyvari-Vigh, A. Miyata, K. Mizuno, D. H. Coy, and C. Kitada.** Tissue distribution of PACAP as determined by RIA: Highly abundant in the rat brain and testes. *Endocrinology* 129: 2787-2789, 1991.

2. **Arimura, A., and S. Shioda.** Pituitary adenylate cyclase activating polypeptide (PACAP) and its receptors: neuroendocrine and endocrine interaction. *Front. in Neuroendocrinol.* 16: 53-88, 1995.

3. **Braas, K. M, and V. May.** Pituitary adenylate cyclase-activating polypeptides, PACAP-38 and PACAP27, regulation of sympathetic neuron catecholamine, and neuropeptide Y expression through activation of type I PACAP/VIP receptor isoforms. *Ann. NY Acad. Sci.* 805: 204-216, 1996.

4. **Cardenas, A. M., C. Montiel, C. Esteban, R. Borges, and A. G. Garcia.** Secretion from adrenaline- and noradrenaline-storing adrenomedullary cells is regulated by a common dihydropyridine-sensitive calcium channel. *Brain Res.* 456: 364-366, 1988.

5. **Champion, H. C., J. A. Santiago, E. A. Garrison, D. Y. Cheng, D. H. Coy, W. A. Murphy, R. J. Ascuitto, and P. J. Kadowitz.** Analysis of cardiovascular responses to PACAP-27, PACAP-38, and vasoactive intestinal polypeptide. *Ann. NY Acad. Sci.* 805: 429-442, 1996.

6. **De Champlain, J., and H. Eid.** The sympathetic system in the pathogenesis of hypertension. *Jpn. Circ. J.* 2: 119-132, 1989.

7. **Gandia, L., R. Borges, A. Albillos, and A. G. Garcia.** Multiple calcium channel

subtypes in isolated rat chromaffin cells. *Pflugers Arch - Eur. J. Physiol.* 430: 55-63, 1995.

8. **Gaspo, R., L. Lamarche, J. de Champlain, and N. Yamaguchi.** Canine adrenal catecholamine response to VIP is blocked by PACAP6-27 in vivo. *Am. J. Physiol.*272 (*Regulatory Integrative Comp. Physiol.* 41): R1606-R1612, 1997.

9. **Gaspo, R., N. Yamaguchi, and J. de Champlain.** Effects of nifedipine and BAY K 8644 on stimulation-induced adrenal catecholamine secretion in the dog. *Am. J. Physiol.* 265 (*Regulatory Integrative Comp. Physiol.* 34): R28-R34, 1993.

10. **Gottschall P. E., I. Tatsuno, A. Miyata, and A. Arimura.** Characterization and distribution of binding sites for the hypothalamic peptide pituitary adenylate cyclase activating polypeptide. *Endocrinology* 127: 2772-277, 1990.

11. **Guo, X., and A. R. Wakade.** Differential secretion of catecholamine in response to peptidergic and cholinergic transmitters in rat adrenals. *J. Physiol. (Lond.)* 475:539-545, 1994.

12. **Ishizuka, Y., K. Kashimoto, T. Mochizuki, K. Sato, K. Ohshima, and N. Yanaihara.** Cardiovascular and respiratory actions of pituitary adenylate cyclase-activating polypeptides. *Reg. Peptide* 40: 29-39, 1992.

13. **Isobe K., T. Nakai, and Y. Takuwa.** Ca^{2+}-dependent stimulatory effect of pituitary adenylate cyclase-activating polypeptide in catecholamine secretion from cultured porcine adrenal medullary chromaffin cells. *Endocrinology* 132: 1757-1765, 1993.

14. Isobe K., N. Yukimasa, T. Nakai, and Y. Takuwa. Pituitary adenylate cyclase-activating polypeptide induces gene expression of the catecholamine synthesizing

enzymes, 3', 5' -cyclic adenosine monophosphate- and protein kinase C-dependent mechanisms in cultured porcine adrenal medullary chromaffin cells. *Neuropeptides* 32 (2): 167-175, 1996.

15. **Kim, S. J., W. Lim, and J. Kim.** Contribution of L- and N-type calcium currents to exocytosis in rat adrenal medullary chromaffin cells. *Brain Res.* 675: 286-296, 1995.

16. **Kimura C., S. Ohkubo, K. Ogi, M. Hosoya, Y. Itoh, H. Onda, A. Miyata, L. Jiang, R. R. Dahl, H. H. Stibbs, A. Arimura, and M. Fujino.** A novel peptide which stimulates adenylate cyclase: Molecular cloning and characterization of the ovine and human cDNAs. *Biochem Biophys. Res. Commun.* 166: 81-89, 1990.

17. **Martineau, D., R. Briand, and N. Yamaguchi.** Functional evidence for L-type Ca^{2+} channels controlling adrenal catecholamine release induced by angiotensin II in vivo. Am. J. Physiol. 271 (*Regulatory Integrative Comp. Physiol.* 40): R1713-R1719, 1996.

18. **Miyata A., A. Arimura, R. R Dahl, N. Minamino, A. Uehara, L. Jiang, M. D. Culler, and D. H. Coy.** Isolation of a novel 38-residue hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. *Biochem. Biophys. Res. Commun. 164*: 567-574, 1989.

19. **Nadeau, R. A., and J. de Champlain.** Plasma catecholamines in acute myocardial infarction. *Am. Heart J.* 98: 548-554, 1979.

20. **Przywara D. A., X. Guo, M. L. Angelilli, T. D. Wakade, and A. R. Wakade.** A non-cholinergic transmitter, pituitary adenylate cyclase activating polypeptide,

utilizes a novel mechanism to evoke catecholamine secretion in rat adrenal chromaffin cells. *J. Bio. Chem.* 271: 10545-10550, 1996.

21. **Runcie, M. J., L. G. Ulman, and E. K. Potter.** Effects of pituitary adenylate cyclase-activating polypeptide on cardiovascular and respiratory responses in anaesthetized dogs. *Reg. Peptid.* 60: 193-200, 1995.

22. **Seki, Y., Y. Suzuki, M. K. Baskaya, K. Saito, M. Takayasu, M. Shibuya, and K. Sugita.** Central cardiovascular effects induced by intracisternal PACAP in dogs. *Am. J. Physiol.* (*Heart Circ. Physiol.* 38): H135-H139, 1995.

23. **Tanaka, K., I. Shibuya, T. Nagamoto, H. Yamashita, and T. Kanno.** Pituitary cyclase-activating polypeptide causes rapid Ca^{2+} release from intracellular stores and long lasting influx mediated by Na^{+} influx-dependent membrane depolarization in bovine adrenal chromaffin cells. *Endocrinology* 137: 956-966, 1996.

24. **Wakade A. R., X. Guo, R. Strong, A. Arimura, and J. Haycock.** Pituitary adenylate cyclase-activating polypeptide (PACAP) as a neurotransmitter in rat adrenal medulla. *Regul. Pep.* 37: 331, 1992.

25. **Watanabe, T., Y. Matsumoto, N. Suzuki, T. Ohtaki, Y. Masuda, C. Kitada, M. Tsuda, and M. Fujino.** Pituitary adenylate cyclase activating polypeptide provokes cultured rat chromaffin cells to secrete adrenaline. *Biochem. Biophys. Res. Commum.* 182: 403-411, 1992.

26. **Watanabe, T., N. Shimamoto, A. Takahashi, and M. Fujino.** PACAP stimulates catecholamine release from adrenal gland medulla: a novel noncholinergic secretagogue. *Am. J. Physiol.* 269 (*Endocrinol. Metab.*32): E903-E909, 1995.

27. **Yamaguchi, N.** In vivo evidence for adrenal catecholamine release mediated by nonnicotinic mechanism: local medullary effect of VIP. *Am. J. Physiol.* 265 (*Regulatory Integrative Comp. Physiol.* 34): R766-R771, 1993.

28. **Yamaguchi, N.** Implication of L-type Ca^{2+} channels in noncholinergic adrenal catecholamine secretion by endothelin-1 in vivo. *Am. J. Physiol.* 269 (*Regulatory Integrative Comp. Physiol.* 38): R287-R293, 1995.

29. **Yon, L., N. Chartrel, M. Feuilloley, S. de Marchis, A. Fournier, E. de Rijk, G. Pelletier, E. Roubos, and H. Vaudry.** Pituitary adenylate cyclase-activating polypeptide stimulates both adrenocortical cells and chromaffin cells in the frog adrenal gland. *Endocrinology* 135: 2749-2758, 1994.

30. **Yonezawa, T., Y. Furukawa, M. Lakhe, Y. Nagashima, M. Hirose, and S. Chiba.** PACAP-38 activates parasympathetic nerves in isolated, blood-perfused dog atria. *Eur. J. Pharmacol.* 315: 289-296, 1996.

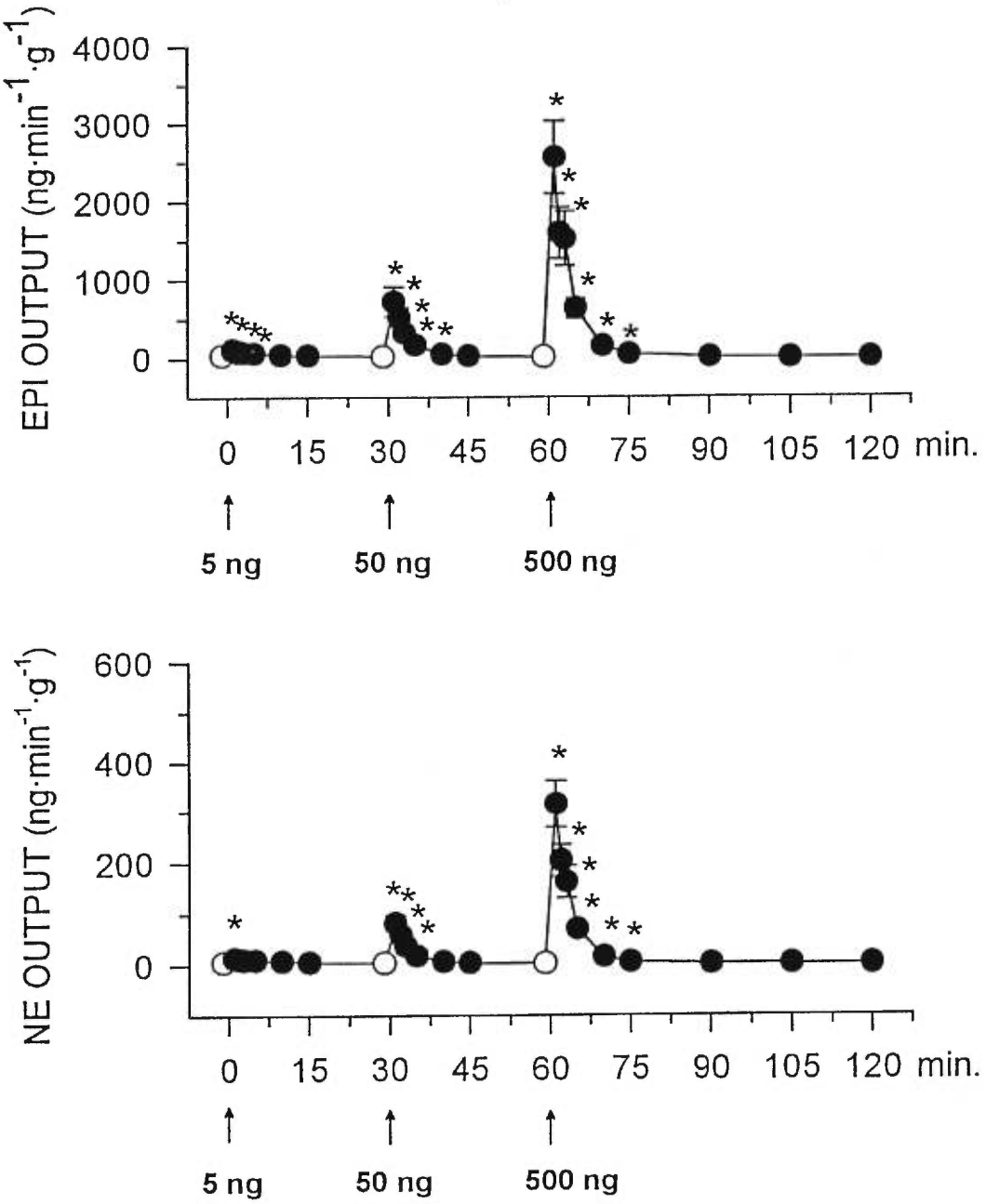

Fig. 2.1. Adrenal epinephrine (EPI, upper panel) and norepinephrine (NE, lower panel) output in response to local infusion of PACAP27 with doses of 5, 50, and 500 ng given at 0, 30, and 60 min, respectively. Arrows indicate time of administration. * $P < 0.05$ vs. the corresponding control value indicated by an open circle.

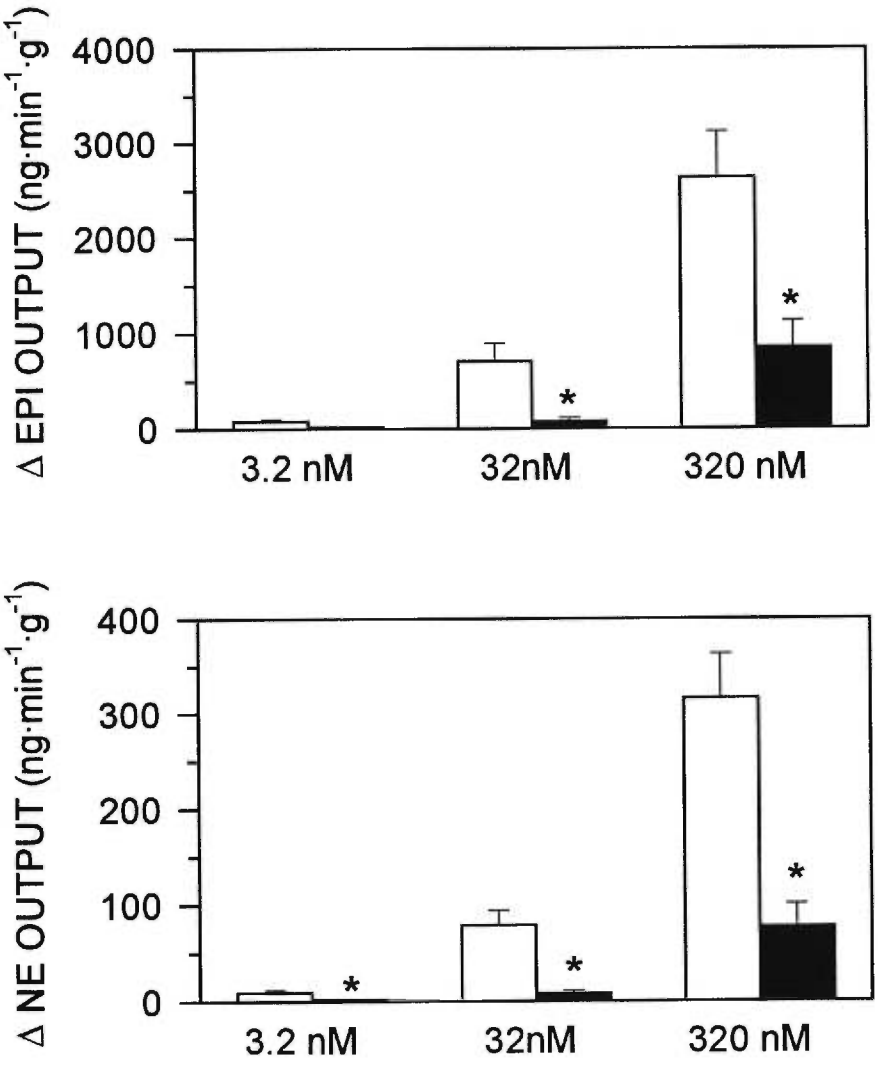

Fig. 2.2. Maximum increases in adrenal epinephrine (ΔEPI, upper panel) and norepinephrine(ΔNE, lower panel) output in response to various doses of PACAP27 (open columns) and PACAP38 (filled columns). *, $P < 0.05$ vs. the corresponding response obtained with PACAP27.

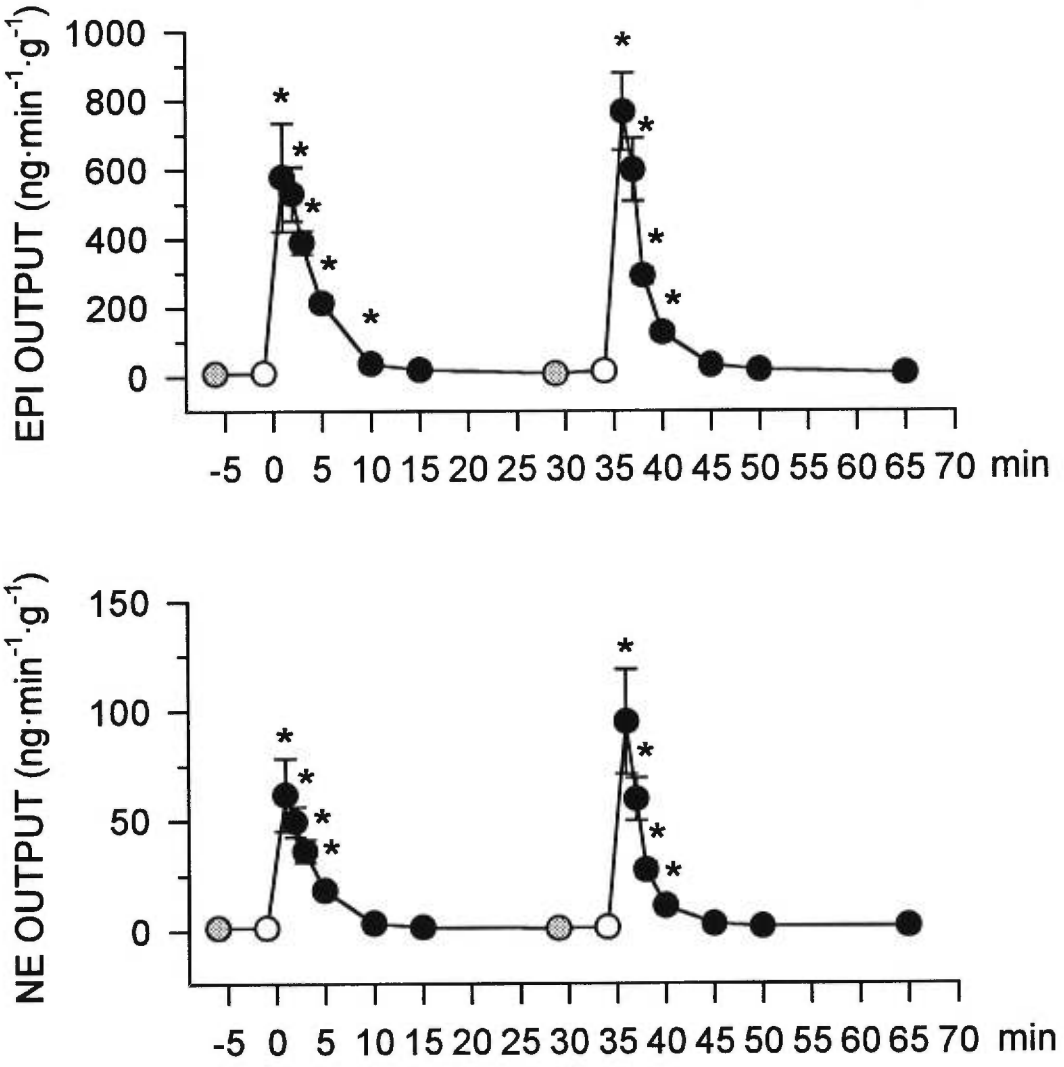

Fig. 2.3. Adrenal epinephrine (EPI, upper panel) and norepinephrine (NE, lower panel) output in response to repeated administration of PACAP27 (50 ng) at 0 and 35 min in the vehicle control group. First and second vehicle was administered at -5 and 30 min, respectively. The stippled circle represents control value observed immediately before the vehicle administration. *, $P < 0.05$ vs. the corresponding control value indicated by an open circle.

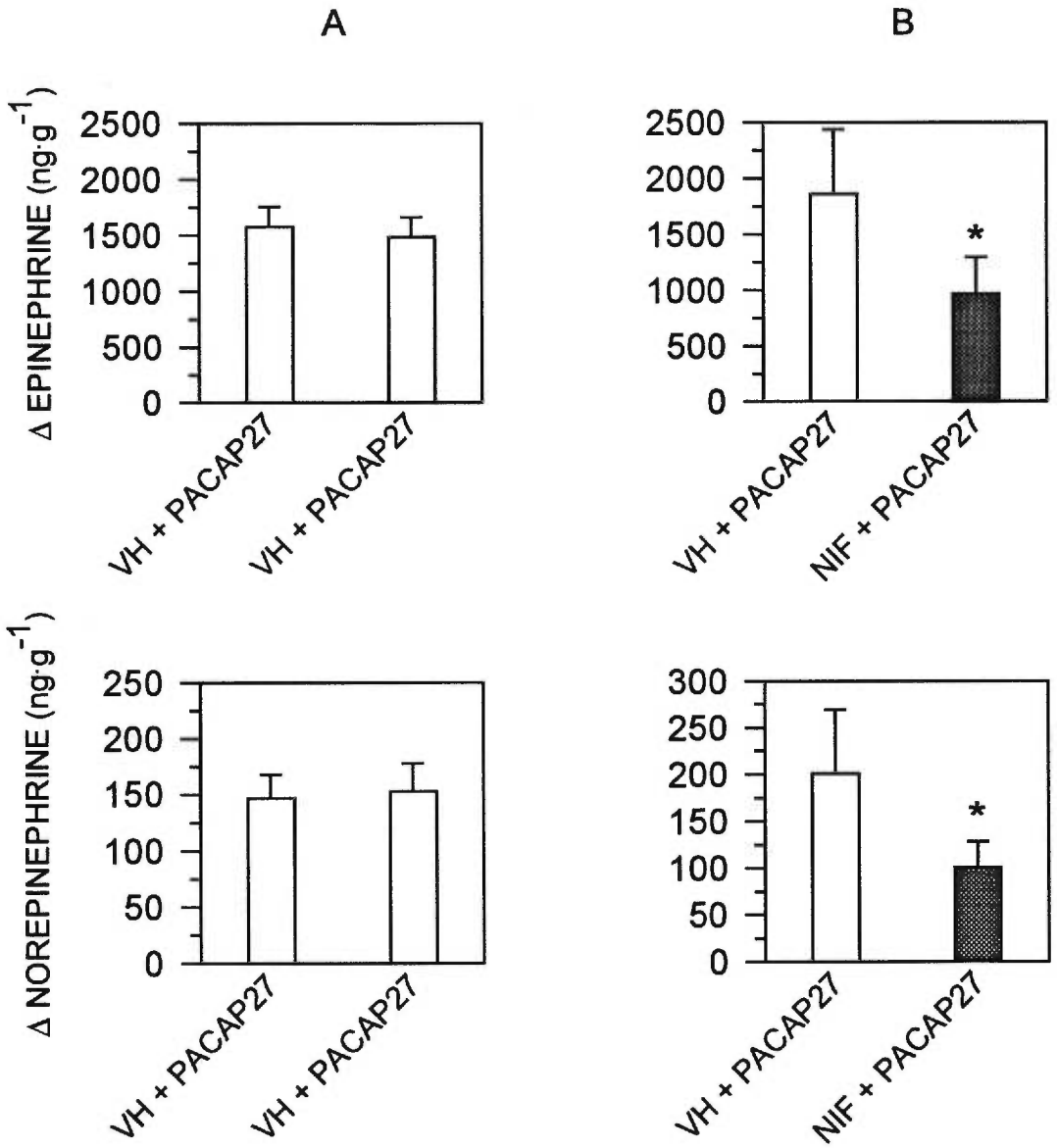

Fig. 2.4. Net amount of epinephrine (upper panel) and norepinephrine (lower panel) released during the first 5 min after the onset of PACAP27 (50 ng) infusion in the vehicle (VH) control group (coloumn A) and in the group receiving nifedipine (NIF, 50 μg, coloumn B). *, $P < 0.05$ vs. the corresponding vehicle control value. The original data are shown in Figures 3 and 5.

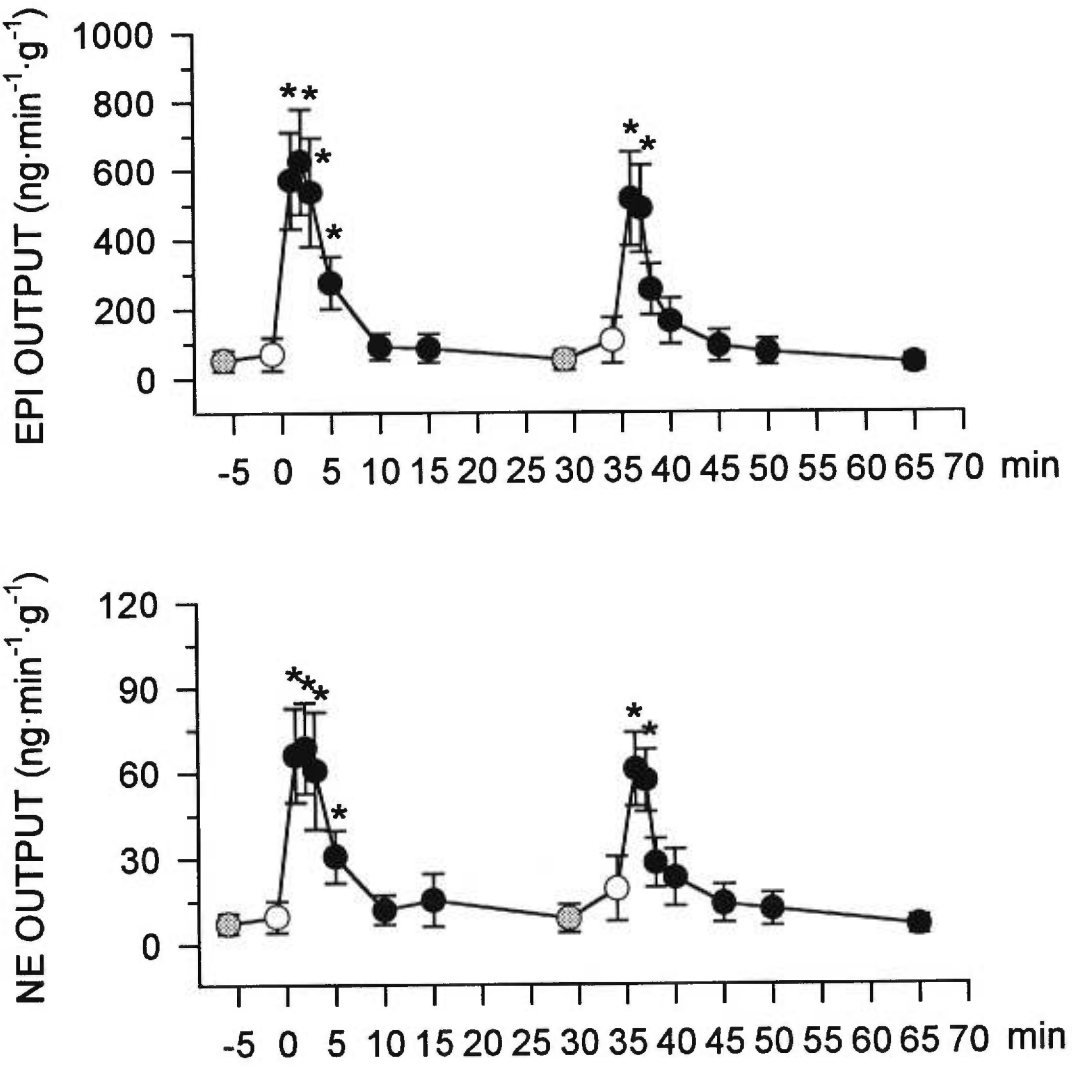

Fig. 2.5. Adrenal epinephrine (EPI, upper panel) and norepinephrine (NE, lower panel) output in response to repeated administration of PACAP27 (50 ng) at 0 and 35 min in the absence and presence of nifedipine (50 μg), respectively. The vehicle and nifedipine was given at -5 and 30 min, respectively. The stippled circle represents control value observed immediately before the administration of either vehicle or nifedipine. *, $P < 0.05$ vs. the corresponding control value indicated by an open circle.

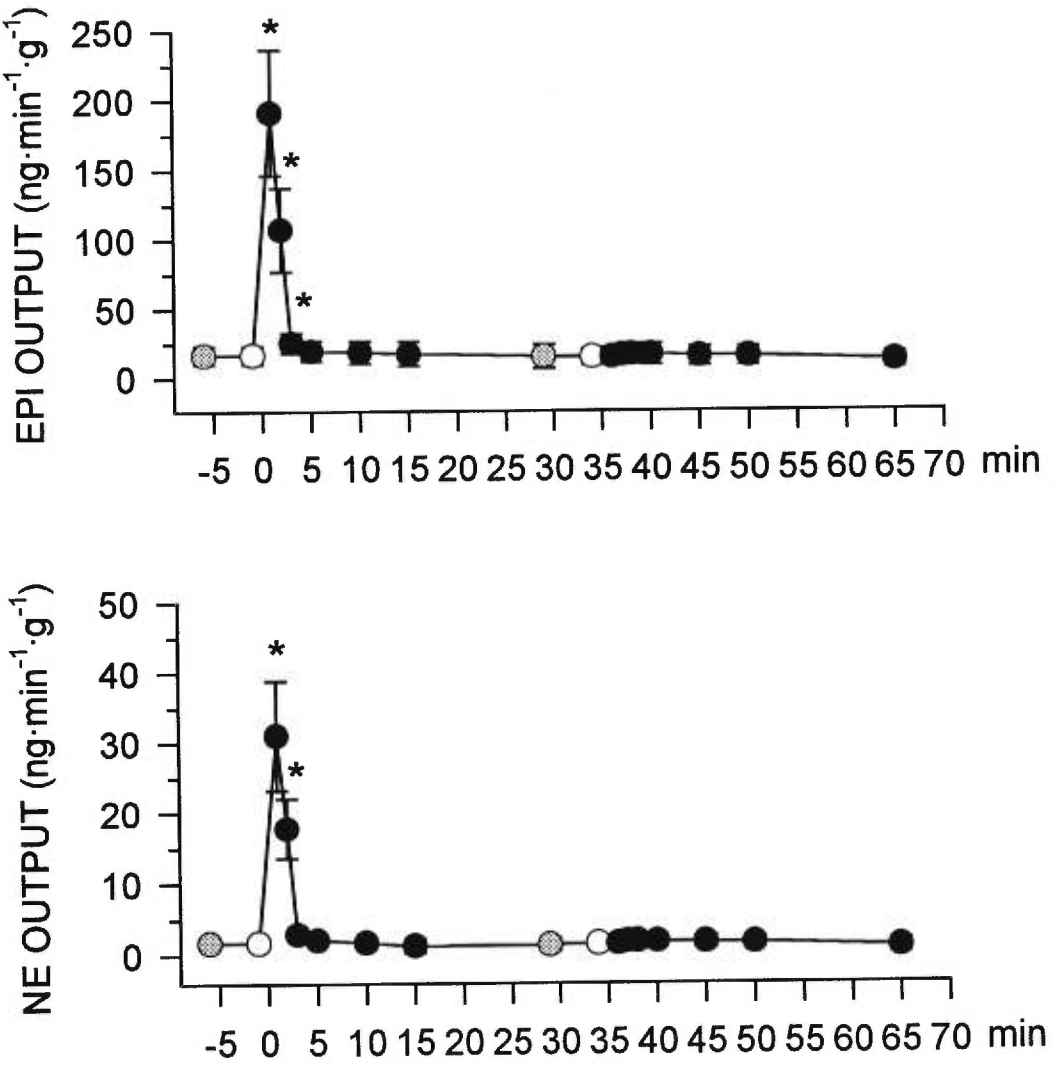

Fig. 2.6. Adrenal epinephrine (EPI, upper panel) and norepinephrine (NE, lower panel) output in response to repeated administration of BAY-K 8644 (5 μg) at 0 and 35 min in the absence and presence of nifedipine (50 μg), respectively. The vehicle and nifedipine was administered at -5 and 30 min, respectively. The stippled circle represents control value observed immediately before the administration of either vehicle or nifedipine. *, $P <$ 0.05 vs. the corresponding control value indicated by an open circle.

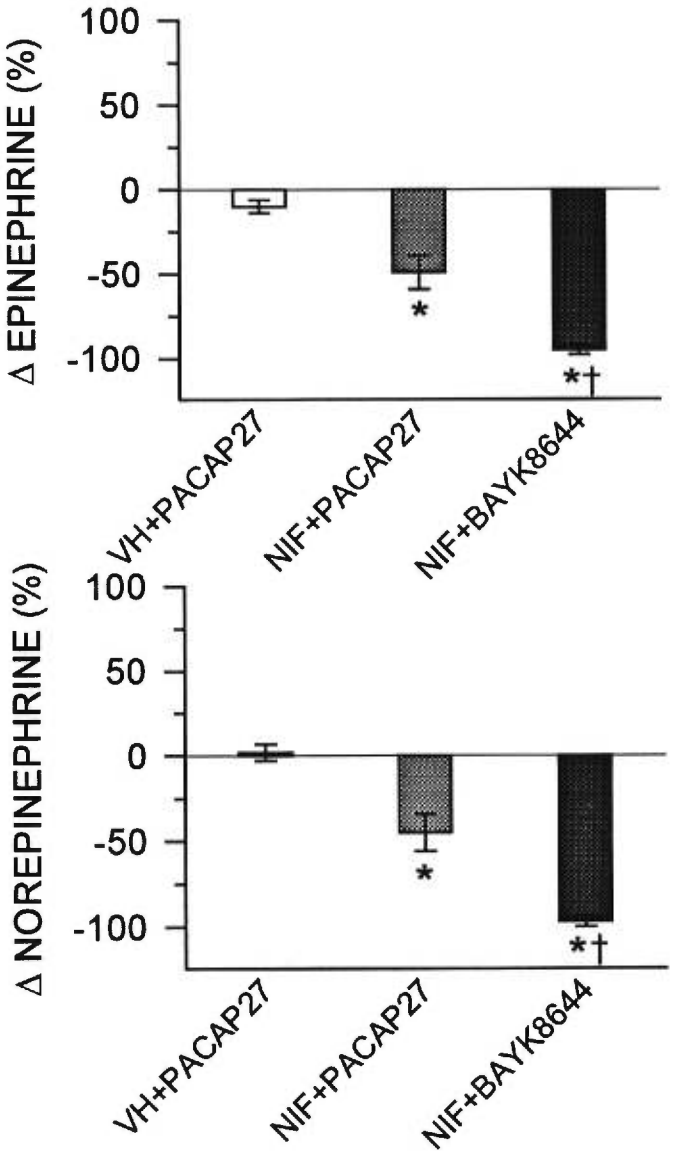

Fig. 2.7. Percent changes in the net amount of epinephrine (upper panel) and norepinephrine (lower panel) released during the first 5 min after the onset of the infusion of either PACAP27 (50 ng) or BAY-K 8644 (5 μg) in the vehicle (VH) control group and in the group receiving nifedipine (NIF, 50 μg). *, $P < 0.05$ vs. VH + PACAP27 group; †, $P < 0.05$ vs. NIF + PACAP27 group. The original data are shown in Figures 3, 5 and 6.

CHAPTER 3

GENERAL DISCUSSION AND CONCLUSIONS

3.1. GENERAL DISCUSSION

3.1.1. Methodology-an in vivo model

The principal objective of the present study was to investigate local effects of PACAP on the basal secretion of adrenal catecholamines in anesthetized dogs. A previous study has indicated that intravenous administration of PACAP induces either hypotension associated with tachycardia or hypertension associated with bradycardia (Ishizuka et al. 1992, Runcie et al. 1995). Such severe changes in systemic blood pressure may significantly affect the sympathoadrenal output through indirect reflex mechanisms most probably via baroreflex (Yamaguchi 1992). To avoid undesired systemic effects such as this, an in vivo dog model previously developed in our laboratory (Yamaguchi 1993) was used in the present study. In this model, local effects of any given drug on the adrenal gland can be evaluated without being disturbed by their undesired potential systemic effects (Yamaguchi 1993). As observed in present study, local administration of PACAP significantly increased adrenal catecholamine secretion, but circulating catecholamine levels in aortic blood remained unchanged. Moreover, neither mean aortic pressure nor heart rate changed significantly during PACAP infusion. These findings are consistent with the view that the model used in the present study was a suitable one to investigate local effects of PACAP and related drugs on the adrenal gland in vivo without their undesired potential systemic effects.

3.1.2. The mechanisms of PACAP-induced adrenal catecholamine secretion

Previous studies have shown that PACAP is present in adrenal medulla where it may

play a role as a noncholinergic neurotransmitter. Both PACAP and ACh induced adrenal catecholamine release in rat chromaffin cells and in anesthetized rats (Watanabe et al. 1992, 1995, Chowdhury et al. 1994). However, the mechanism of catecholamine release in response to PACAP is different from that to ACh. PACAP caused a long-term increase in adrenal catecholamine secretion, whereas ACh did not (Watanabe et al. 1992). The PACAP-induced adrenal catecholamine release was not inhibited by treatment with the cholinergic antagonists mecamylamine and atropine, but ACh-induced adrenal catecholamine release was significantly inhibited by such cholinergic antagonists (Watanabe et al. 1995). Furthermore, DMPP (1,1-dimethyl-4-phenylpiperazinium, a ganglionic stimulating drug) and PACAP both release large amounts of Leu-EK (enkephalin) and catecholamines in bovine adrenal chromaffin cells. DMPP caused a fast and effective action, while PACAP caused a slow and more sustained action (Babinski et al. 1996). It has been suggested that the interactive control of catecholamine secretion was mediated by cholinergic and peptidergic receptors (Chowdhury et al. 1994). Our experimental results also suggested that PACAP directly induced adrenal medullary catecholamine release in anesthetized dogs. Taken together, these results suggested that PACAP directly induces adrenal catecholamine secretion, and not indirectly via cholinergic mechanisms.

It is well accepted that both Ca^{2+} influx and Ca^{2+} release from intracellular Ca^{2+} store play a role as the major mechanism of intracellular Ca^{2+} increase in adrenal chromaffin cells. Ca^{2+} influx could be through several types of voltage-dependent Ca^{2+} channels (Gandia et al. 1995). L-type voltage-dependent Ca^{2+} channel is a major route for Ca^{2+} entries (Cena et

al. 1989). Ca^{2+} release from intracellular Ca^{2+} store was sensitive to IP3 or caffeine (Stauderman et al. 1991). PACAP could induce increases of intracellular Ca^{2+} concentration and catecholamine secretion from several cultured adrenal chromaffin cells (Tanaka et al. 1996, Isobe et al. 1992, Przywara et al. 1996). Present study shows that L-type Ca^{2+} channel blocker inhibited PACAP-induced catecholamine release, but it did not completely abolish this effect in anesthetized dogs. It has also been suggested that PACAP causes Ca^{2+} release from intracellular Ca^{2+} stores in bovine and porcine adrenal chromaffin cells (Tanaka et al. 1996, Isobe et al. 1992). The caffeine (ryanodine)-sensitive Ca^{2+} stores are the major source of PACAP-induced Ca^{2+} release from intracellular Ca^{2+} store, and IP3 dependent Ca^{2+} stores may play a minor role in PACAP-induced Ca^{2+} release from intracellular Ca^{2+} store (Tanaka et al. 1996). Nevertheless, it appears likely that the Ca^{2+} release from the intracellular stores plays only a minor role in regulation of catecholamine secretion from adrenal chromaffin cells (Tanaka et al. 1996). It seems, therefore, likely that PACAP arises the intracellular Ca^{2+} concentration mainly by increasing Ca^{2+} influx and, secondary, by releasing Ca^{2+} from intracellular Ca^{2+} stores.

PACAP38 is at least 100-fold more potent than PACAP27 in stimulating IP3 turnover. However, both PACAP27 and PACAP38 have similar potencies on cAMP accumulation (Spengler et al. 1993). In present study, PACAP27 was about 3 to 5 fold more potent than PACAP38 in adrenal catecholamine secretion in anesthetized dogs. This observation is compatible with the idea that PACAP27-induced catecholamine secretion is due to the cAMP production, and not due to an increase in IP3 turnover.

PACAP increases intracellular cAMP levels in rat adrenal chromaffin cell (Watanabe et al. 1992, Przywara et al. 1996). In comparison with VIP, PACAP was approximately 100 to 1000 times more potent in increasing intracellular cAMP concentration and in evoking catecholamine secretion (Watanabe et al. 1992). An increase in intracellular cAMP is known to facilitate the secretion of catecholamines in rat adrenal medulla (Malhotra et al. 1989). However, intracellular cAMP alone is not sufficient to trigger catecholamine secretion. Intracellular Ca^{2+} increase is necessary to initiate the exocytosis of catecholamines (Malhotra et al. 1989). It has been suggested that PACAP-induced catecholamine release results from an increase in cAMP-dependent Ca^{2+} influx (Przywara et al. 1996). In this context, the present data obtained with nifedipine support the role of Ca^{2+} influx as a major mechanism in PACAP-induced catecholamine release. However, the observation that nifedipine only partially inhibited the catecholamine response to PACAP suggests the existence of additional mechanisms operating in the canine adrenal in vivo.

There exist at least two types of PACAP receptors, type I (PACAP-specific site) and type II (PACAP and VIP shared site), characterized on the basis of the relative affinities for PACAP and VIP (Arimura and Shioda 1995, Harmar and Lutz 1994, Shivers et al. 1991). The adrenal medulla is one of tissues containing high density of PACAP type I receptors, which recognize PACAP much more selectively than VIP (Spengler et al. 1993, Watanabe et al. 1995). It has been suggested that PACAP-induced catecholamine secretion from adrenal medulla was mediated by type I receptor-mediated mechanisms (Watanabe et al. 1995). In this context, it is of particular interest that, in the similar experimental approach

in anesthetized dogs, VIP-induced adrenal catecholamine release was significantly inhibited by PACAP6-27, a selective PACAP type I receptor antagonist (Gaspo et al. 1997). Furthermore, it appears likely that the increase in catecholamine release through a dihydropyridine-resistant mechanism is closely related to the cyclic AMP-mediated Ca^{2+} influx (Przywara et al. 1996). Moreover, a recent study indicated that PACAP stimulated the production of both cAMP and inositol phosphates in bovine adrenal chromaffin cells (Babinski et al. 1996). Taken together, it can be speculated that PACAP27-induced adrenal catecholamine secretion may results from the stimulation of PACAP type I receptor, which, in turn, activates both adenylate cyclase and phospholipase C, resulting in increases in cyclic AMP-dependent Ca^{2+} entry and Ca^{2+} release from intracellular Ca^{2+} stores. The functional involvement of the PACAP type I receptor and cyclic AMP- and inositol phosphates-mediated mechanisms in adrenal catecholamine secretion in vivo remains to be demonstrated in future studies.

3.1.3. Future investigation

The present results indicated that L-type Ca^{2+} channels play a role in PACAP-induced adrenal catecholamine secretion. However, various mechanisms related to the Ca^{2+} influx remains to be elucidated in future studies.

It would be of particular importance to verify if PACAP type I receptor is functionally involved in PACAP-induced catecholamine release under in vivo conditions.

Adrenal catecholamine release in response to PACAP needs to be examined in the presence of PACAP6-27 or PACAP6-38, selective PACAP receptor antagonists. If adrenal catecholamine response to PACAP is actually mediated by the specific PACAP receptor, the presence of these antagonists may abolish the response.

According to findings in vitro, the formation of cAMP was enhanced by PACAP. However, it remains unclear whether PACAP increases plasma cAMP levels in adrenal venous blood or tissue adenylate cyclase activity. Forskolin is a potent activator of adenylate cyclase and increases cAMP formation in vitro (Seamon and Daly 1983). ACh-evoked catecholamine release has been shown to be markedly potentiated by the pretreatment with forskolin. This observation suggests that cAMP may play a role in the modulation of catecholamine release from bovine chromaffin cells (Morita et al. 1987). Therefore, it would be of interest to know whether forskolin can enhance the PACAP-induced catecholamine release under in vivo conditions. Thus, the investigation of the role of cAMP in PACAP-induced catecholamine release would be necessary.

3.2. CONCLUSION

The present study led to the following conclusions:

Adrenal catecholamine secretion significantly increased following the local administration of PACAP to the left adrenal gland, while plasma catecholamine levels in

aortic blood remained unchanged, indicating the direct local drug effect on the adrenal medulla in vivo. PACAP is thus a potent secretagogue of catecholamine in anesthetized dogs. Both PACAP27 and PACAP38 directly stimulate the medulla resulting in an increase in catecholamine release in a dose-dependent manner. PACAP27-induced catecholamine release is about 3 to 5 fold greater than that induced by PACAP38 on the same molar basis. Nifedipine significantly blocked by about 50% the PACAP27-induced catecholamine release, indicating that L-type Ca^{2+} channels are operative, at least in part, in PACAP-induced catecholamine release in the canine adrenal gland in vivo.

REFERENCES

Akaike, A., Y. Mine, M. Sasa, and S. Takaori. Voltage and current clamp studies of muscarinic and nicotinic excitation of the rat adrenal chromaffin cells. *J. Pharmacol. Exp. Ther.* 255: 333-339, 1990.

Arimura, A., A. Somogyvari-Vigh, A. Miyata, K. Mizuno, D. H. Coy, and C. Kitada. Tissue distribution of PACAP as determined by RIA: Highly abundant in the rat brain and testes. *Endocrinology.* 129:2787-2789, 1991.

Arimura, A., A. Somogyvari-Vigh, C. Weill, R. C. Fiore, I. Tatsuno, V. Bay, and D. E. Brenneman. PACAP functions as a neurotrophic factor. *Ann. N.Y. Acad. Sci.* 739: 228-243, 1994.

Arimura, A., and S. Shioda. Pituitary adenylate cyclase activating polypeptide (PACAP) and its receptors: neuroendocrine and endocrine interaction. *Front. Neuroendocrinol.* 16: 53-58, 1995.

Axelrod, J., and R. Weinshiboum. Catecholamines. *N. Engl. J. Med.* 287: 237, 1972.

Babinski, K., V. Bodart, M. Roy, A. De Léan, and H. Ong. Pituitary adenylate-cyclase activating polypeptide (PACAP) evokes long-lasting secretion and de novo biosynthesis of bovine adrenal medullary neuropeptides. *Neuropeptides.* 30: 572-582, 1996.

Bargmann, W. Über die neurosekretorische verknüpfung von hypothalamus und neurohypophyse. *Z. Zell-forsch.* 34: 610-634, 1949.

Bargmann, W., E. Lindner, and K. H. Andres. Uber synapsen an endokrinen epithelzellen und die definition sekretorischer neurone. Untersuchungen am zwischenlappen der katzenhypophse. *Z. Zell-forsch.* 77: 282-298, 1967.

Bauer F. E., G. W. Hacker, G. Terenghi, T. E. Adrian, J. M. Polak, and S. R. Bloom. Localization and molecular forms of galanin in human adrenals: elevated levels in pheochromocytomas. J. *Clin Endocrinol Metab.* 63: 1372-1378, 1986.

Bean, B. P. Classes of calcium channels in vertebrate cells. *Annu. Rev. Physiol.* 51:367-384. 1989.

Bloom, S. R., and J. M. Polsk. Control of pancreatic hormone release by islet innervation. *In Current views on hypoglycaemia and glucagon.* eds: D. Andreani, P. J. Lefebvre, and V. Marks. Academic Press, London, Toronto, pp. 37-46, 1980

Boarder, M. R., and D. B. Marriott. Endothelin-1 stimulation of noradrenaline and adrenaline release from adrenal chromaffin cells. *Biochem. Pharmacol.* 41: 521-526, 1991.

Boksa, P., and B. G. Livett. Substance P protects against desensitization of the nicotinic response in isolated adrenal chromaffin cells. *J. Neurochem.* 42: 619-627, 1984.

Boonyaviroj, P., and Y. Gutman. Acetylcholine and cAMP in adrenal medulla: Indirect effect. *Naunyn-Schmiedeberg's Arch Pharmacol.* 297: 241-243, 1977.

Boonyaviroj, P., and Y. Gutman. Alpha- and beta-adrenoceptors and PGE2 in the modulation of catecholamine secretion from bovine adrenal medulla in vitro. *J. Phmar. Pharmacol.* 31: 716-717. 1979.

Braaun-Menendez, E., J. C. Fasicolo, L. F. Lelor, and J. M. Munoz. Farmacologia de la hipertensina. *Revista de la Sociedad Argentina de Biologia (Buenos Aires)* 16: 398-410, 1940.

Bredt, D. S., P. M. Hwang and S. H. Snyder. Localization of nitric oxide synthase indicating a neural role for nitric oxide. *Nature (Lond).* 347: 768-770, 1990.

Breslow, M. J. Regulation of adrenal gland medullary and cortical blood flow. *Am. J. Physiol.* 262: H1317-H1330, 1992.

Brown, A. M. and L. Birnbaumer. Direct G protein gating of ion channels. *Am. J. Physiol.* 254: H401-H410, 1988.

Brownstein, M. J., and B. J. Hoffman. Neurotransmitter transporters. *Recent. Prog. Horm. Res.* 49: 27-42, 1994.

Bruhn, T. O., W. C. Engeland, E. L. P. Anthoy, D. S. Gann, and I. M. D. Jackson. Corticotrophin-releasing factor in the dog adrenal medulla is secreted in response to hemorrhage. *Endocrinology* 120: 25-33, 1987.

Bucsics, A., and F. Lembeck. In vitro release of substance P from spinal cord slices by capsaicin congeners. *Eur. J. Pharmacol.* 71: 71-77, 1981.

Burgoyne, R. D. Control of exocytosis in adrenal chromaffin cells. *Biophysical Acta* 1071: 174-202, 1991.

Burgoyne, R. D., A. Morgan, I. Robinson, N. Pender, and T. R. Cheek. Exocytosis in adrenal chromaffin cells. *J. Anat.* 183: 309-314, 1993.

Campbell, K. P., A. T. Leng, and A. H. Sharp. The biochemistry and molecular biology of the dihydropyridine-sensitive calcium channel. *Trend Neurosci.* 11: 425-430, 1988.

Cannon, W. B., and J. E. Uridil. Studies on the conditions of activity endocrine glands.

iiiv. Some effects on the denervated heart of stimulating the nerves of the liver. *A. J. Physiol.* 58: 353-354, 1921.

Cannon, W. B., and A. Rosenbleuth. In "Autonomic Neuroeffector systems" MacMillan, New York. 1937.

Cardell, L. O., R. Uddman, A. Luts, and F. Sundler. Pituitary adenylate cyclase activating peptide PACAP in guinea-pig lung: distribution and dilatory effects. *Regul. Pep.* 36: 379-390, 1991.

Cardenas, A. M., C. Montiel, A. G. Garcia, P. Michelena, and P. Sanchez-Garciaa. Mechanism of blockade by (+)isradipine of adrenal catecholamine release. *Eur. J. Pharmacol.* 192: 243-251, 1991.

Cena, V., A. Stutzin, and E. Rojas. Effects of calcium and BayK-8644 on calcium currents in adult bovine chromaffin cells. *J. Membr. Biol.* 112: 255-265, 1989.

Champion H. C., J. A. Santigo, E. A. Garrison, D. Y. Cheng, D. H. Coy, W. A. Murphy, R. J. Ascuitto, N. T. Ross-Ascuitto, D. B. McNamara, and P. J. Kadowitz. Analysis of cardiovascular responses to PACAP-27, PACAP-38, and vasoactive intestinal polypeptide. *Anna. N.Y. Acad. Sci.* 805: 429-441, 1996.

Chasserot-Golaz, S., N. Vitale, I. Sagot, B. Delouche, S. Dirrig, L. A. Pradel, J. P. Henry, D. Aunis, and M. F. Bader. Annexin II in exocytosis: catecholamine secretion requires the translocation of p36 to the subplasmalemmal region in chromaffin cells. *J. Cell Biol.* 133: 1217-1236, 1996.

Chowdhury, P. S., X. Guo, T. D. Walade, D. A. Przywara, and A. R. Wakade. Exocytosis from a single rat chromaffin cell by cholinergic and peptidergic neurotransmitters. *Neuroscience.* 59: 1-5, 1994.

Christophe, J. Type I receptors of PACAP (a neuropeptide even more important than VIP?). *Biochem. Biophys. Acta.* 1154: 183-199, 1993.

Cotecchia, S., D. A. Schwinn, R. R. Randall, R. J. Lefkowitz, M. G. Caron, and B. K. Kobilka. Molecular cloning and expression of the cDNA for the hamster α1-adrenergic receptor. *Porc. Natl. Acad. Sci. USA* 85: 7159-7163, 1988.

Cotecchia, S., B. K. Kobilka, K. W. Daniel, R. D. Nolan, E. C., Lapetina, M. G. Caron, R. J. Lefkowitz, and J. W. Regan. Multiple second messenger pathways of α-adrenergic receptor subtypes expressed in eukaryotic cells. *J. Biol. Chem.* 265: 63-69, 1990.

Coupland, R. E. The chromaffin system. In *Catecholamines, Handbook of Experimental*

pharmacology. eds: H. Blaschko, E. Muschool. Berlin, Springer-Verlag, 33: 16-39, 1972.

Coy, D. H., N. Y. Jiang, J. Fuselier, and W. Murphy. Structural simplification of potent growth hormone-releasing hormone analogs: implication for other members of the VIP/GHRH/PACAP family. *Ann. N.Y. Acad. Sci.* 805: 149-158, 1996.

Cryer, P. E. The adrenal medulla. ed: V. T. James. *The adrenal gland.* Paven press. 1992.

Dun, N. J., H. Tang, S. L. Dun, R. Huang, E. C. Dun, and A. R. Wakade. Pituitary adenylate cyclase activating polypeptide-immunoreactive sensory neurons innervate rat adrenal medulla. *Brain Research* 716: 11-21, 1996.

Dunning, B. E., P. J. Havel, R. C. Veith, and G. J. Taborsky, Jr. Pancreatic and extrapancreatic galanin release during sympathetic neural activation. *Am. J. Physiol.* 258 (*Endocrinoi. Metab.* 21): E436-E444, 1990.

Eccles, J. C., P. Fatt, and K. Koketsu. Cholinergic and inhibitory synapses in a pathway from motoraxon collaterals to motoneurones. *J. Physio.* 126: 524-562, 1954.

Emorine, L. J., S. Marullo, M-M. Briend-Sutren, K. Tate, C. Delavier-Klutchko, and D. Stros-berg. Molecular characterization of the human β3-adrenergic receptor. *Science*. 245:1118-1121, 1989.

Fahrenkrug, J. Vasoactive intestinal peptide: measurement, distribution and putative transmitter function. *Digestion* 19: 149-169. 1979.

Fahrenkrug, J. Vasoactive intestinal peptide (VIP) and autonomic neurotransmission. In: *Novel Peripheral Neurotransmitters*, ed: C. Bell. Toronto: Pergamon, p. 113-133. 1991.

Foucart, S., R. Nadeau, and J. de Champlain. The release of catecholamines from the adrenal medulla and its modulation by α2-adrenoceptors in the anesthetized dog. *Can. J. Physiol. Pharmacol.* 65: 550-557, 1987.

Foucart, S., R. Nadeau, and J. De Champlain. Local modulation of adrenal catecholamines release by β2-adrenoceptors in the anesthetized dog. *Nnaunyn-Schmiedeberg`s Arch. Pharmacol.* 337: 29-34, 1988.

Fridolf, T., F. Sundler, and B. Ahren. Pituitary adenylate cyclase-activating peptide (PACAP): Occurrence in rodent pancreas and effects on insulin and glucagon secretion in the mouse. *Cell Tissue Res.* 269: 275-279, 1992.

Frödin, M., J. Hannibal, B.S. Wulff, S. Gammeltoft and J. Fahrenkrug. Neuronal localization of pituitary adenylate cyclase-activating polypeptide 38 in the adrenal medulla

and growth-inhibitory effect on chromaffin cells. *Neuroscience*. 65: 599-608, 1995.

Gandia, L., M. G. Lopez, R. I. Fonteriz, C. R. Artalejo, and A. G. Garcia. Relative sensitivities of chromaffin cell calcium channels to organic and inorganic calcium antagonists. *Neurosci. Lett.* 77: 333-338, 1987.

Gandia, L., R. Borges, A. Albillos, and A. G. Garcia. Multiple calcium channel subtypes in isolated rat chromaffin cells. *Pflugers Arch-Eur J. Physiol.* 430: 55-63, 1995.

Garcia, A. G., F. Sala, J. A. Reig, S. Viniegra, J. Frias, R. Fonteriz, and L. Gandia. Dihydropyridine BAY-K-8644 activates chromaffin cell calcium channels. *Nature (Lond).* 309: 39-71, 1984.

Garrido, B., F. Abad, and A. G. Garcia. Modulation by voltage of calcium channels and adrenal catecholamine release. *Ann. NY Acad. Sci.* 635: 459-463, 1991.

Gaspo, R., N. Yamaguchi, and J. de Champlain. Effects of nifedipine and BAY K 8644 on stimulation-induced adrenal catecholamine secretion in the dog. *Am. J. Physiol.* 265 (*Regulatory Integrative Comp. Physiol.* 43): R28-R34, 1993.

Gaspo, R., L. Lamarche, N. Yamaguchi, and J. de Champlain. Nifedipine inhibits adrenal but not circulating catecholamine response to nicotinic stimulation in dogs. *Am. J. Physiol.* 267 (*Regulatory Integrative Comp. Physiol.* 36): R1545-R1551, 1994.

Gaspo, R., N. Yamaguchi, and J. de Champlain. Correlation between neural release of VIP and adrenomedullary catecholamine secretion in vivo. *Am. J. Physiol.* 268 (*Regulatory Integrative Comp. Physiol.* 37): R1449-R1455, 1994.

Gaspo, R., L. Lamarche, J. de Champlain, and N. Yamaguchi. Canine adrenal catecholamine response to VIP is blocked by PACAP-(6-27) in vivo. *Am. J. Physiol.* 272(*Regulatory Integrative Comp. Physiol.* 41): R1606-R1612, 1997.

Goldfien, A. Adrenal Medulla. *In Basic & clinical endocrinology*. 4th edition, eds: Greenspan, F. S., and J. D. Baxter. Appleto & Lunge. pp.370-385, 1994.

Goodman, L. S, and A. Gilman. (eds). *The pharmacological basis of therapeutics,* 9th edition, Macwila Publishing, NewYork, 1996.

Granneman, J. G., K. N. Lahners, and A. Chsudhry. Characterization of the human β3-adrenergic receptor gene. *Mol. Pharmacol.*, 44: 264-270, 1993.

Gulbenkian, S., J. Wharton, G. W. Hacher, I. M. Varndell, S. R. Bloom and J. M. Polak. Co-localization of neuropeptide tyrosine (NPY) and its C-terminal flanking peptide

(C-PON). *Peptides* 6: 1237-1243. 1985.

Gutierrez, L. M., J. M. Canaves, A. V. Ferrer-Montiel, J. A. Reig, M. Montal, and S. Viniegra. A peptide that mimics the carboxy-terminal domain of SNAP-25 block Ca^{2+}-dependent exocytosis in chromaffin cells. *FEBS Letters.* 372: 39-43, 1995.

Harmar, A., and E. Lutz. Multiple receptors for PACAP and VIP. *Trends Pharmacol. Sci.* 15: 97-99, 1994.

Heinsimer, J. A, and R. J. Lefkowitz. Adrenergic receptors: biochemistry, regulation, molecular mechanism, and clinical implications. *J. Lab. Clin. Med.* 100: 641-658, 1982.

Hess, P. Calcium channels in vertebrate cells. *Annu. Rev. Neurosci.* 13: 337-358, 1990.

Hinning, L. D., A. P. Fox, E. W. McCleskey, B. M. Olivera, S. A. Thayer, R. J. Miller and R. W. Tsien. Dominant role of N-type Ca^{2+} channels in evoked release of norepinephrine from sympathetic neurons. *Science Wash. DC* 239: 57-61, 1988.

Hokfelt, T., J. M. Lundberg, M. Schultzberg, and J. Fahrenkrug. Immunohistochemical evidence for a local VIP-ergic neuron system in the adrenal gland of the rat. *Acta Physiol. Scand.* 113: 575-576, 1981.

Houchi, H., M. Oka, M. Misbahuddin, K. Morita, and A. Nakanishi. Stimulation by vasoactive intestinal polypeptide of catecholamine synthesis in isolated bovine adrenal chromaffin cells. *Biochem. Pharmacol.* 36: 1551-1554, 1987.

Hughes. J., and G. N. Woodruff. Neuropeptides. Function and clinical applications. *Arzneimittel - Forschung.* 42: 250-255, 1992.

Insel, P.A. Identification and regulation of adrenergic receptors in target cells. *Am. J. Physiol.* 247: E53-E58, 1984.

Ishihara, T., R. Shigemoto, K. Mori, K. Takahashi, and S. Nagata. Functional expression and tissue distribution of a novel receptor for vasoactive intestinal polypeptide. *Neuron* 8: 811-819, 1992.

Ishizuka, Y., K. Kashimoto, T. Mochizuki, K. Sato, K. Ohshima, and N. Yanaihara. Cardiovascular and respiratory actions of pituitary adenylate cyclase-activating polypeptides. *Regul. Pept.* 40: 29-39. 1992.

Isobe, K., T. Nakai and Y. Takuwa. Ca^{2+}-dependent stimulatory effect of pituitary adenylate cyclase-activating polypeptide on catecholamine secretion from cultured porcine adrenal medullary chromaffin cells. *Endocrinology.* 132: 1757-1765. 1993.

Johnson, R. G. Accumulation of biological amines into chromaffin granules: a model for hormone and neurotransmitter transport. *Physiol Rev.*68: 232-307, 1988.

Junqueira, L. C., J. Carneiro, and R. O. Kelley. (eds). *Basic Histology*. 7th edition, Appleton &Lange. Cinnecticut. 1992.

Kandel, E., and T. Abel. Neuropeptide, adenylyl cyclase, and memory storage. *Science* 268: 825-826, 1995.

Kastin, A. J., J. E. Zadina, R. D. Olson, and W. A. Banks. The history of neuropeptide research: version 5.a. *Ann. NY Acad. Sci.* 780: 1-18, 1996.

Katsoulis, S., A. Clemens, H. Schwöere, W. Creutzfeldt, and W. E. Schmidt. Pituitary adenylate cyclase-activating peptide is a potent relaxant of the rat ileum. *Peptides* 14: 587-592, 1993.

Kawai, K., C. Ohse, Y., Watanabe, S. Suzuki, K. Yamashita and S. Ohashi. Pituitary adenylate cyclase activating peptide stimulates insulin release from isolated perfused rat pancreas. *Life Sci.* 50: 257-261, 1992.

Kee, Y., R. C. Lin, S. C. Hsu, and R. H. Scheller. Distinct domains of syntaxin are required for synaptic vesicle fusion complex formation and dissociation. *Neuron.* 14: 991-998, 1995.

Kessler, J. A, J. E. Adler, and I. B. Black. Substance P and somatostatin regulate sympathetic noradrenergic function. *Science.* 221: 1059-1061, 1983.

Khalil, Z., P. Z. Marley, and B. G. libett. Elevation in plasma catecholamines in response to insulin stress in under both neuronal and non-neuronal control. *Endocrinology (Baltimore)*. 119: 159-167, 1986.

Khalil, Z., B. G. Livett, and P. D. Marley. Sensory fibres modulate histamine-induced catecholamine secretion from the rat adrenal medulla and sympathetic nerves. *J. Physiol. (London)*, 391: 511-526, 1987.

Khalil, Z., P. D. Marley, and B. G. Livett. Effect of substance P on nicotine-induced desensitization of cultured bovine adrenal chromaffin cells: possible receptor subtypes. *Brain Res.* 495, 282-288, 1988.

Kimura, C., S. Ohkubo, S., K. Ogi, N. Hosoya, Y. Itoh, H. Onda, A. Miyata, L. Jiang, R. R. Dahl, H. H. Stibbs, A. Arimura, and M. Fujino. A novel peptide which stimulates adenylate cyclase: Molecular cloning and characterization of the ovine and human cDNAs.

Biochem. Biophys. Res. Commun. 166: 81-89, 1990.

Kimura, T., T. Shimamura, and S. Satoh. Effects of pirenzepine and hexamethonium on adrenal catecholamine release in response to endogenous and exogenous acetylcholine in anesthetized dogs. *J. Cardiovasc. Pharmacol.* 20: 870-874, 1992.

Kirpekar, S. M., and M. Puig. Effect of flow-stop on noradrenaline release from normal spleen, and spleens treated with cocaine, phentolamine or phenoxybenzamine. *Br. J. Pharmac.* 43: 359-369, 1971.

Kopin, I. J. Metabolic degradation of catecholamines. The relative important of different pathways under physiological and after administration of drugs. In, *Catecholamines.* (Blaschko, H. K. F., and Muscholl, E., eds) Handbuch der Expermentellen Pharmakologie. *Springer-Verlag, Berlin.*33: 271-282, 1972.

Kovacs, C. S., C. L. Chik, B. Li, E. Karpinski, and A. K. Ho. Pituitary adenylate cyclase-activating-peptide stimulates cyclic AMP accumulation in UMR 106 osteoblast-like cells. *J. Endocrinology.* 149: 287-295, 1996.

Kryvi, H. Comparison of the ultrastructure of adrenaline and noradrenaline storage granules of bovine adrenal medulla. *Eur. J. Cell Biol.* 20: 76, 1979.

Kuramoto, H., H. Kondo, and T. Fujita. Neuropeptide tyrosine (NPY)-like immunoreactivity in adrenal chromaffin cells and intraadrenal nerve fibers of rats. *Anat. Rec.* 214: 321-328, 1986.

Kuramoto, H., H. Kondo, and T. Fujita. Calcitonin gene related peptide (CGRP)-like immunoreactivity in scattered chromaffin cells and nerve fibers in the adrenal gland of rats. *Cell Tissue Res.* 247: 309-315, 1987.

Landsberg, L., and J. B. Young . Catecholamines and the adrenal medulla. 7th ed. eds: Wilson and Foster. *Textbook of endocrinology.* W.B. Saunders company. 891-965, 1985.

Leeman, S. E. Substance P and neurotensin: Discovery, isolation, chemical characterization and physiological studies. *J. Exp. Biol.* 89: 193-200, 1980.

Leonard, B. E. Inter-relationship between neurotransmitters. *Neuropharmacology* 23: 213-218, 1984.

Linden, A., S. Yoshihara, B. Chan, and J. A. Nadel. Inhibition of bronchoconstriction by pituitary adenylate cyclase activating polypeptide (PACAP 1-27) in guinea-pigs in vivo. *Br. J. Pharmacol.* 115: 913-916, 1995.

Linnoila, R. I., R. P. Diaugustine, A. Hervonen, and R. J. Miller. Distribution of (Met5) and (Leu5)-enkephalin, vasoactive-intestinal polypeptide and substance P-like immunoreactives in human adrenal glands. *Neuroscience.* 5: 2470-2259, 1980.

Lipscombe, D., D. V. Madison, M. Poenie, H. Reuter, R. Y. Tsien, and R. W. Tsien. Spatial distribution of calcium channels and cytosolic calcium transients in growth cones and cell bodies of sympathetic neurons. *Proc. Natn. Acad. Sci. U.S.A.* 85: 2398-2402, 1988.

Lipscombe, D., S. Kongsamut, and R. W. Tsien. Alpha-adrenergic inhibition of sympathetic neurotransmitter release mediated by modulation of N-type calcium-channel gating. *Nature* 340: 639-642, 1989.

Livett, B. G, F. Kozobe F, D. and M. Dean. Substance P inhibits nicotinic activation of chromaffin cells. *Nature.* 278: 256-257, 1979.

Livett, B. G, P. Boksa, D. M. Dean, F. Mizobe, and M. H. Lindenbaum. Use of isolated chromaffin cells to study basic release mechanisms. *J. Auton. Nerv. Syst.* 7: 59-86, 1983.

Livett, B. G., and P. D. Marley. Noncholinergic control of adrenal catecholamine secretion. *J. Anat.* 183: 277-289, 1993.

Loewi, O. Uber humoral Uebertragbarleit der Herznervenwirkung. *Pflugers Arch Ges Physio* 189: 239-242, 1921.

Lopez, M. G. M. A. Moro, C. F. Castillo, C. R. Artalejo, and A. G. Garcia. Variable, voltage-dependent, blocking effects of nitrendipine, verapamil, diltiazem, cinnarizine and cadmium on adrenomedullary secretion. *Br. J. Pharmacol.* 96: 725-731, 1989.

Lopez, M. G., A. Alibillos, M. T. de la Fuente, R. Borges, L. Gandia, E. Carbone, A. G. Garcia, and A. R. Artalejo. Localized L-type calcium channels control exocytosis in cat chromaffin cells. *Pfkugers Archiv - Europwan J. Physiol.* 427: 348-354, 1994.

Lundberg, J. M., and T. Hökfelt. Coexistence of peptides and classical neurotransmitters. *Trends Neurosci.* 6: 325-333, 1983.

Maas, J. W., H. Benensohn, and H. D. Landis: A kinetic study of the disposition of circulating norepinephrine in normal male subjects. *J. Pharmacol. Exp. Ther.* 174: 381, 1970.

MacNulty, E. E., S. J. McClue, I. C. Carr, T. Jess, M. J. O. Wakelam, and G. Milligan. α2-C10 adrenergic receptors expressed in Rat 1 fibroblasts can regulate both adenylylcyclase and phospholipase D-mediated hydrolysis of phosphatidylcholine by interacting with pertussis toxin-sensitive guanine nucleotide-binding proteins. *J. Biol. Chem.*

267: 2149-2156. 1992.

Mains, R. E., A. Zhou, and D. Parkinson. The biosynthetic processing and secretion of endogenous carboxypeptidase H in mouse pituitary cells. *Ann. N. Y. Acad. Sci.* 805: 10-18, 1996.

Majane, E. A, H. Alho, Y. Katokoa, C. H. Lee, and H. Y. Yang. Neuropeptide Y in bovine adrenal glands: distribution and characterization. *Endocrinology.* 117: 1162-1168, 1985.

Malhotra, R. K., T. D. Wakade, and A. R. Wakade. Vasoactive intestinal polypeptide and muscarine mobilize intracellular Ca^{2+} through breakdown of phosphoinositides to induce catecholamine secretion. Role of IP3 in exocytosis. *J. Biol. Chem.* 263: 2123-2126, 1988(a).

Malhotra, R. K., T. D. Wakade, and A. R. Wakade. Cross-communication between acetylcholine and VIP in controlling catecholamine secretion by affecting cAMP, inositol trisphosphate protein kinase c, and calcium in rat adrenal medulla. *J. Neurosicience.* 9: 4250-4157, 1989.

Marley, P. D. New insights into the non-nicotinic regulation of adrenal medullary function. *Trends Pharmacol. Sci.* 8: 411-413, 1987.

Martineau, D., N. Yamaguchi, and R. Briand. Inhibition by BMS 186295, a selective nonpeptide AT1 antagonist, of adrenal catecholamine release induced by angiotensin II in the dog in vivo. *Can. J. Physiol. Pharmacol.* 73: 459-464, 1995.

Martineau, D., R. Briand, and N. Yamaguchi. Functional evidence for L-type Ca2+ channels controlling ANG- IIinduced adrenal catecholamine release in vivo. *Am. J. Physiol.* 271 (*Regulatory Integrative Comp. Physiol.* 40): R1713-R1719, 1996.

Meldolesi, J., and T. Pozzan. Pathway of Ca^{2+} influx at the plasm membrane: voltage-, receptor-, and second-messenger-operated channels. *Exp. Cell. Res.* 171: 271-283, 1987.

Millhorn, D. E, and T. Hokfelt. Chemical messengers and their coexistence in individual neurons. *News Physiol Sci.* 3: 1-5, 1988.

Minkes, R. K., T. J. Mcmahon, T. R. Higuera, W. A. Murphy, D. H. Coy, and P. J. Kadowitz. Analysis of systemic and pulmonary vascular responses to PACAP and VIP: role of adrenal catecholamines. *Am. J. Physiology.* 263: H1659-H1669. 1992.

Miyata, A., A. Arimura, R. R. Dahl, N. Minamino, A. Uehara, L. Jiang, M. D. Culler, and D. H. Coy. Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates

adenylate cyclase in pituitary cells. *Biochem. Biophys. Res. Commun.* 164: 567-57, 1989.

Miyata, A., L. Jiang, R. D. Dahl, C. Kitada, K. Kubo, M. Fujino, N. Minamino, and A. Arimura. Isolation of a neuropeptide corresponding to the N-terminal 27 residues of the pituitary adenylate cyclase activating polypeptide with 38 residues (PACAP38). *Biochem. Biophys. Res. Commun.* 170: 643-648, 1990.

Mohamed, A. A., T. L. Parker, and R. E. Coupland. The innervation of the adrenal gland. II. The source of spinal afferent nerve fibers to the guinea-pig adrenal gland. *J. Anat.* 160: 51-58, 1988.

Moller, K., and F. Sundler. Expression of pituitary adenylate cyclase activating peptide (PACAP) and PACAP type I receptors in the rat adrenal medulla. *Regul. Pept.* 63: 129-139, 1996.

Montiel, C., A. R. Artalejo, and A. G. Garcia. Effects of the novel dihydropyridine bay K 8644 on adrenomedullary catecholamine release evoked by calcium reintroduction. *Biochem. Biophys. Res. Commun.* 120: 851-857, 1984.

Morita, K., T. Dohi, S. Kitayama, Y. Koyama, and A. Tsujimoto. Enhancement of stimulation-evoked catecholamine release from cultured bovine adrenal chromaffin cells by forskolin. *J. Neurochem.* 48: 243-247, 1987.

Mungan, Z., A. Arimura, A. Ertan, W. J. Rossowski, and D. H. Coy. Pituitary adenylate cyclase-activating peptide relaxes rat gastrointestinal smooth muscle. *Scand. J. Gastroenterol.* 27: 375-380, 1992.

Nakazato, Y., Y. Yamada, U. Tomita, and A. Ohga. Muscarinic agonists release adrenal catecholamine by mobilizing intracellular Ca^{2+}. *Proc. Jpn. Acad.* 60: 314-317, 1984.

Parker, T. L., W. K. Kesse, A. A. Mohamed, and M. Afework. The innervation of the mammalian adrenal gland. *J. Anat.* 183: 265-276, 1993.

Peart, W. S. The nature of splenic sympathin. *J. Physiol (Lond)* 108: 491-501, 1949.

Perlman, R. L, and M. Chalfie. Catecholamine release from the adrenal medulla. *Clin Endocrinal Metab* 6: 551-576, 1977.

Piascik, M. T., E. E. Soltis, M. M. Piascik and L. B. Macmillan. α-Adrenoceptors and vascular regulation: molecular, pharmacologic and clinical correlates. *Pharmacol. Ther.* 72: 215-241, 1996.

Pollet, R. J, and G. S. Levey. Principles of membrane receptor physiology and their

application to clinical medicine. *Ann Intern Med* . 92: 663-680, 1980.

Przywara, D. A., X. Guo, M. L. Angelilli, T. D. Wakade, and A. R. Wakade. A non-cholinergic transmitter, pituitary adenylate cyclase-activating polypeptide, Utilizes a novel mechanism to evoke catecholamine secretion in rat adrenal chromaffin cells. *J. Biolo. Chem.* 271: 10545-10550, 1996.

Rand, M. J., H. Majewski, H. Wong-Dusting, D. F. Story, R. E. Loia, and J. Ziogas. Modulation of neuroeffector transmission. *J. Cardiovasc. Pharmacol.* 10(Suppl. 12): S33-S44, 1987.

Rand, M. J., H. Majewski, and D. F. Story . Modulation of neuroeffector transmission. In *Cardiovascular Pharmacology.* ed. M. Antonaccio. Raven Press, New York, pp 229-292, 1990.

Rawlings, S. R. PACAP, PACAP receptors, and intracellular signalling. *Mol. Cell. Endocrinol.* 101: C5-C9, 1994.

Robberecht, P. M. C. Woussen-Colle, P. De Neef, P. Gourlet, L. Buscail, A. Vandermeers, and M. C. Vandermeers-Piret. The two forms of the pituitary adenylate cyclase activating polypeptide (PACAP(1-27) and PACAP(1-38)) interact with distinct receptors on rat pancreatic AR 4-2J cell. *FEBS lett.* 286: 133-136, 1991.

Robberecht, P., P. Gourlet, P. De Neef, M-C. Woussen-colle, M-C. Vandermeers-Piret, A. Vandermeers and J. Christophe. Receptor occupancy and adenylate cyclase activation in AR 4-2J rat pancreatic acinar cell membranes by analogs of pituitary adenylate cyclase-activating peptides amino-terminally shortened or modified at position 1, 2, 3 , 20, or 21. *Am. Soc. Pharmacol. Exp. Ther.* 42: 347-355, 1992.

Roth, J., and Le Roith, D. Intercellular communication: The evolution of scientific concepts and of messenger molecules. *In Medicine, Science, and Society.* Symposia celebrating the Harvard Medical School Bicentennial, ed: K. J. Isselbacher, New York, Wiley. pp: 425-447.1984.

Runcie, M. J., L. G. Ulman, and E. K. Potter. Effects of pituitary adenylate cyclase-activating polypeptide on cardiovascular and respiratory responses in anaesthetised dogs. *Regul. Pep.* 60: 193-200, 1995.

Rundle, S. E., B. J. Canny, P. M. Robinson, and J. W. Funder. Innervation of the sheep adrenal cortex: an immunohistochemical study with rat corticotrophin-releasing factor antiserum. *Neuroendocrinology* 48: 8-15, 1988.

Saavedra, J. M., H. Grobecher, and J. Axelrod. Adrenaline-forming enzyme in

brainstem: elevation in genetic and experimental hypertension. *Science* 191: 438-484, 1975.

Said, S. I., and V. Mutt. Potent peripheral and splanchnic vasodilator peptide from normal gut. *Nature* 225: 863-865, 1970.

Sawangjaroen, K., C. R. Dallemagne, R. B. Cross, and J. D. Curlewis. Effect of pituitary adenylate cyclase activating polypeptide (PACAP) and vasoactive intestinal polypeptide (VIP) on the cardiovascular system in sheep. *Peptides* 13: 1029-1032, 1992.

Schneider, A. S, H. L. Cline, and S. Lemaire. Rapid rise in cycle GMP accompanies catecholamine secretion in suspensions of isolated adrenal chromaffin cells. *Life Sci* 24: 1389-1394, 1979.

Schneider, A. Muscarinic receptor mechanism in adrenal chromaffin cells. *In Stimulus-Secretion Coupling in Chromaffin Cells.* ed: K. Rosenheck and P. I. Lelkes. Boca Raton, CRC Press. PP.51-86.1987.

Schwörer, H., S. Katsoulis, W. Creutzfeldt, and W. E. Schmidt. Pituitary adenylate cyclase activating peptide, a novel VIP-like gut-brain peptide, relaxes the guinea-pig taenia caeci via apamin-sensitive potassium channels. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 346: 511-514, 1992.

Schwörer, H., A. Clemens, S. Katsoulis, H. Köhler, W. Creutzfeldt, and W. E. Schmidt. Pituitary adenylate cyclase activating peptide is a potent modulator of human colonic motility. *Scand. J. Gastroenterol.* 28: 625-632, 1993.

Seamon, K. B., and J. W. Daly. Forskolin, cyclic AMP and cellular physiology. *Trends Pharmacol. Sci.* 4: 120-123, 1983.

Seebeck, J., W. E. Schmidt, H. Kilbinger, J. Neumann, N. Zimmermann, and S. Herzig. PACAP induces bradycardia in guinea-pig heart by stimulation of atrial cholinergic neurones. *Naunyn-Schmiedebergs Archives of Pharmacology.* 354: 424-430, 1996.

Shah, S. D., T. F. Tse, W. E. Clutter, and P. E. Cryer. The human sympathochromaffin system. *Am. J. physiolo.* 247: E380-384, 1984.

Sharman, D. F. The catebolism of catecholamine: recent studies. *Br. Med. Bull.* 29: 110-115, 1973.

Shiotani, T., S. Kimura, Y. Ohshige, C. Yanaihara, and N. Yanihara. Immunohistochemical localization of pituitary adenylate cyclase-activating polypeptide (PACAP) in the adrenal medulla of the rat. *Peptides.* 16: 1045-1050, 1995.

Shivers, B. D., T. J. Gorcs, P. E. Gottschall, and A. Arimura. Two high affinity binding sites for pituitary adenylate cyclase-activating polypeptide have different tissue distributions. *Endocrinology* 128: 3055-3065, 1991.

Smith, A. D. Cellular control of the uptake, storage and release of noradrenaline in sympathetic nerves. *Biochem Soc Symp.* 36: 103-131, 1972.

Spengler, D., C. Waeber, C. Pantaloni, F. Holsboer, J. Bocker, P. H. Seeburg, and L. Journot. Differential signal transduction by five splice variants of the PACAP receptor. *Nature (Lond).* 365: 170-175. 1993.

Stauderman, K. A., and R. M. Pruss. Dissociation of Ca^{2+} entry and Ca^{2+} mobilization responses to angiotensin II in bovine adrenal chromaffin cells. *J. Biol. Chem.* 264: 18349-18355, 1989.

Stauderman, K. A., and R. M. Pruss. Dissociation of Ca^{2+} entry and Ca^{2+} mobilization responses to angiotensin II in bovine adrenal chromaffin cells. *J. Biol. Chem.* 264: 18349-18355, 1989.

Steer, M. L. Adrenergic receptors. *Clin. Endocrinol. Metab.* 6: 577-595, 1977.

Stjarne, L. Recent observation on the regulation of storage and release of catecholamines. *Research Publications-Association for Research in Nervous & Mental Disease.* 43: 325-342, 1966.

Stjarne, L. The synthesis, uptake and storage of catecholamines in the adrenal medulla: the effect of drugs. In: *Catecholamines. Handbook of Experimental Pharmacology.* eds. Blaschko H, Muscholl E. Berlin: Springer-Verlag, 33: 231-269, 1972.

Starke, A. M., W. B. Sawyer, L. M. Marubio, and A. D. Loewy. Spinal origin of sympathetic preganglionic neurons in the rat. *Brain Res.* 491: 156-162, 1988.

Starke, A. M., W. B. Sawyer, J. J. Hughes, K. B. Platt, and A. B. Loewy. A general pattern of CNS innervation of the sympathetic outflow demonstrated by transneuronal pseudorabies viral infections. *Brain Res.* 491: 156-162, 1989a.

Strack, A. M., W. B. Sawyer, K. B. Platt, and A. D. Loewy. CNS cell groups regulating the sympathetic outflow to adrenal gland as revealed by transneuronal cell body labeling with pseudorabies virus. *Brain Res.* 491: 274-296, 1989b.

Suzuki, Y., K. Kasai, K. Takekoshi, M. Oka, N. Banba, T. Numao, H. Sugimura, M. Iizuka, and S. Shimoda. Effects of pituitary adenylate cyclase activating polypeptide (PACAP) on the cardiovascular system. *Regu. Pep.* 47: 213-220, 1993.

Sundler, F., Ekblas, E., Bottcher, G., Alumets, J. and Hakanson R. Coexistence of peptides in the neuroendocrine system. In: *Biogenetics of Neurohormonal Peptides*, eds: Hakanson, R. and Thorell, J., pp. 213-243. 1985.

Sweatt, J. D., S. L. Johnson, E. J. Cragoe, and L. E. Limbird. Inhibitions of Na^+/H^+ exchange block stimulus-provoked arachidonic acid release in human platelets: Selective effects on platelet activation by epinephrine, ADP, and lower concentrations of thrombin. *J. Biol. Chem.* 260: 12910-12919, 1985.

Sweatt, J. D., T. M. Connolly, E. J. Grogoe, L. E. and Limbird. Evidence that Na^+/H^+ exchange receptor-mediated phospholipase A2 activation in human platelets. *J. Biol. Chem.* 261: 8667-8673, 1986.

Tabarin, A., D. Chen, R. Hakanson, and F. Sundler. Service adenylate cyclase-activating peptide in the adrenal gland of mammals: distribution, characterization and responses to drugs. *Neuroendocrinology.* 59: 113-119, 1994.

Tanaka, K., I. Shibuya, T. Nagamoto, H. Yamashita, and T. Kanno. Pituitary cyclase-activating polypeptide cause rapid Ca^{2+} release from intracellular store and long lasting influx mediated by Na^+ influx-dependent membrane depolarization in bovine adrenal chromaffin cells. *Endocrinology.* 137: 956-966. 1996.

Tatemoto, K., M. Carlquist, and V. Mutt. Neuropeptide Y - a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. *Nature* 269: 659-660, 1982.

TerBush, D. R, M. A. Bittner and R. W. Holz . Ca^{2+} influx causes rapid translocation of protein kinase c to membranes. Studies of the effects of secretagogues in adrenal chromaffin cells. *J. Biol. Chem.* 263: 18873-18879, 1988.

Thayer, S. A., L. D. Hirning, and R. J. Miller. Distribution of multiple types of Ca^{2+} channels in rat sympathetic neurons in vitro. *Moles. Pharmac.* 32: 579-586, 1987.

Tipton, K. F. Biochemical aspects of monoamine oxidase. *Br. Med. Bull.* 29: 116-119, 1973.

Trabelsi, and F., N. Yamaguchi. Modulation by PACAP of norepinephrine release induced by hepatic nerve stimulation in vivo. *The Liver and Nervous System* (Falk Symposium No 103), Freiburg, Germany. October, 1997. Abstract.

Tsien, R. W., P. T. Ellinor, and W. A. Horne. Molecular diversity of voltage-dependent Ca^{2+} channels. *Trends Pharmacol.* 12:349-354. 1991.

Tuominen, B. K, M. K. McMillian , H. Ye, M. K. Stachowiak, P. M. Hudson, and J. S. Hong. Long-term activation of protein kinase c by nicotine in bovine adrenal chromaffin cells. *J. Neurochem.* 58: 1652-1658, 1992.

Vandermeers, A., S. Vandenborre, X. Hou, P. De Neef, P. Robberecht, M-C. Vandermeers-Piret and J. Christophe. Antagonistic properties are shifted back to agonistic properties by further N-terminal shortening of pituitary adenylate-cyclase-activating peptides in human neuroblastoma NB-OK-1 cell membranes. *Eur. J. Biochem.* 208: 815-819, 1992.

Von Euler, U. S., and J. H. Gaddum. An unidentified depressor substance in certain tissue extracts. *J. Physiol.* 72: 74-87, 1931.

Von Euler, U. S. The presence of a substance with sympathin-E properties in spleen extracts. *Acta Physiol Scand* 11: 168-186, 1946a.

Von Euler, U. S. A specific sympathomimetic ergone in adrenergic nerve fibers (Sypathin) and its relation to adrenaline and noradrenaline. *Acta Physion Scand* 12: 73-79, 1946b.

Von Euler, U. S. The presence of sympathomimetic substance in extracts of mammalian heart. *J. Physiol. (Lond)* 105: 38-44, 1946c.

Wakade, A. R., and T. D. Wakade . Contribution of nicotinic and muscarinic receptors in the secretion of catecholamines evoked by endogenous and exogenous acetylcholine. *Neuroscience*, 10: 973-978, 1983.

Wakade, T. D., A. B. Marion, K. M. Ravindra, P. Roberta, and A. R. Wakade. The peptide VIP is a neurotransmitter in rat adrenal medulla: physiological role in controlling catecholamine secretion. *J. physiol.* 444: 349-362, 1991.

Wakade, A. R., X. Guo, R. Strong, A. Arimura, and J. Haycock. Pituitary adenylate cyclase-activating polypeptide (PACAP) as a neurotransmitter in rat adrenal medulla (Abstract). *Regul. Pept.* 37: 327, 1992.

Wakade, A. R., D. A. Przywara, S. V. Bhave, P. S. Chowdhury, A. Bhave, and T. D. Wakade. Massive exocytosis triggered by sodium-calcium exchange in sympathetic neurons is attenuated by co-culture with cardiac cells. *Neuroscience.* 55: 813-821, 1993.

Warashina, A., N. Fujwara. and K. Shimoji. Characteristics of nicotinic and muscarinic secretory responses in the rat adrenal medulla studies by real-time monitoring of catecholamine release. *Biomed. Res.* 10: 157-164, 1989.

Warren, J. B., J. R. Cockroft, S. W. Lakin, R. Kajekar, A. Macrae, M. A. Ghatei, and

S. R. Bloom. Pituitary adenylate cyclase activating polypeptide is a potent vasodilator in humans. *J. Cardiovasc. Pharmacol.* 20: 83-87, 1992.

Watanabe, T., T. Ohtaki, C. Kitada, M. Tsuda, and M. Fujino. Adrenal pheochromocytoma PC12h cells respond to pituitary adenylate cyclase activating polypeptide. *Biochem. Biophysi. Res. Commun.* 173: 252-258, 1990.

Watanabe, T., Y. Masuo, H. Matsumo, N. Suzuki, T. Ohtaki, Y. Masuda, C. Kitada, M. Tsuda, and M. Fujino. Pituitary adenylate cyclase activating polypeptide provokes cultured rat chromaffin cells to secrete adrenaline. *Bichem. Biophys. Res. Commun.* 182: 403-411, 1992.

Watanabe, T., N. Shimamoto, A. Takahashi, and M. Fujino. PACAP stimulates catecholamine release from adrenal medulla: a novel noncholinergic secretagogue. *Am. J. Physiol.* 269 (*Endocrinol. Metab.* 32): E903-E909, 1995.

Will-Shabab, L., I. Küttner, G. Wallukat, S. Bartel, P. Robberecht, and J. Christophe. 1st international symposium on VIP, PACAP and related regulatory. (Bischenberg, France, September 19-23, 1993). Abstract.

Winkler, H., D. K. Apps, and R. Fischer-Colbrie. The molecular function of adrenal chromaffin granules: established facts and unresolved topics. *Neuroscience.* 18: 261-190, 1986.

Winkler, H, M. Sietzen, and M. Schober. The life cycle of catecholamine-storing vesicles. *Ann NY Acad Sci.* 493:3-19, 1987.

Yada, T., M. Sakurada, M. Nakataa, K. Ihida, K. Yaekura, S. Shioda, and M. Kikuchi. Current status of PACAP as a regulator of insulin secretion in pancreatic islets. *Ann. N.Y. Acad. Sci.* 805: 329-340, 1996).

Yamada, Y., H. Teraoko, Y. Nakazato, and A. Ohga. Intracellular Ca^{2+} antagonist TMB-8 blocks catecholamine secretion evoked by caffeine and acetylcholine from perfused cat adrenal glands in the absence of extracelluar Ca^{2+}. *Neurosci. Lett.* 90: 338-342, 1988.

Yamaguchi, N. Sympathoadrenal system in neuroendocrine control of glucose: mechanisms involved in the liver, pancreas, and adrenal gland under hemorrhagic and hypoglycemic stress. *Can. J. Physiol. Pharmacol.* 70(2): 167-206, 1992.

Yamaguchi, N. Inhibition by nifedipine of endothelin-induced adrenal catecholamine secretion in anesthetized dogs. *Can. J. Physiol. Pharmacol.* 71: 301-305, 1993.

Yamaguchi, N. Implication of L-type Ca^{2+} channels in noncholinergic adrenal

catecholamine secretion by endothelin-1 in vivo. *Am. J. Physiol.* 269 (*Regulatory Intergrative Comp. Physiol.* 38): R287-R293, 1995.

Yamaguchi, N. Presynaptic effect of PACAP27 in the dog pancreas in vivo. *The 8th international catecholamine symposium.* (California, USA, October 13-18, 1996). Abstract.

Yamaguchi, N., F. Trabelsi, G. Geng, and S. Lamouche. PACAP: A neuromodulator in the canine pancreas, liver and adrenal. *The Liver and Nervous System* (Falk Symposium No 103), Freiburg, Germany, October, 1997. Abstract.

Yanagisawa, M., H. Kurihara, S. Kimura, K. Goto, and T. Masaki. A novel peptide vasoconstrictor, endothelin, is produced by vascular endothelium and modulates smooth muscle Ca^{2+} channels. *J. Hypertension - Supp.* 6: S188-S191, 1988.

Yokota, C., K. Kawai, S. Ohashi, Y. Watanabe, and K. Yamachita. PACAP stimulates glucose output from the perfused rat liver. *Peptides.* 16: 55-60, 1995.

Zhang, J-F., A. D. Randall, P. T. Ellinor, W. A. Horne, W. A. Sather, T. Tanabe, T. L. Schwartz, and R. W. Tsien. Distinctive pharmacology and kinetics of cloned neuronal Ca channels and their possible counter parts in mammalian CNS neurons. *Neuropharmacology.* 32: 1075-1088. 1993.

Zhou, X. F., B. J. Oldfield, and B. G. Livett. Substance P-containing sensory neurons in the rat dorsal root ganglia innervate the adrenal medulla. *J. Auton. Nerv. Syst.* 33: 247-254, 1991.