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Mechanism of Pituitary Adenylate Cyclase-Activating Polypeptide-Induced Inhibition on Catecholamine Secretion Evoked by Cholinergic Stimulation and Membrane Depolarization in the Rat Adrenal Gland

Dong-Yoon Lim, Jeong-Won Kang, and Young-Jo Kim[1]

Departments of Pharmacology and [1]Dermatology, College of Medicine, Chosun University, Kwangju 501−759, Korea

The present study was attempted to examine the effect of pituitary adenylate cyclase-activating polypeptide (PACAP) on catecholamine (CA) secretion evoked by cholinergic stimulation, membrane depolarization and calcium mobilization from the isolated perfused rat adrenal gland. The perfusion of PACAP (10 nM) into an adrenal vein for 60 min produced a great inhibition in CA secretion evoked by ACh (5.32×10^{-3} M), high K^+ (5.6×10^{-2} M), DMPP (10^{-4} M for 2 min), McN-A-343 (10^{-4} M for 2 min), cyclopiazonic acid (10^{-5} M for 4 min) and Bay-K-8644 (10^{-5} M for 4 min). Also, in the presence of neuropeptide (NPY), which is known to be co-localized with norepinephrine in peripheral sympathetic nerves, CA secretory responses evoked by ACh, high potassium, DMPP, McN-A-343, Bay-K-8644 and cyclopiazonic acid were also significantly depressed. However, in adrenal glands preloaded with PACAP (10 nM) under the presence of VIP antagonist [(Lys^1, $Pro^{2,5}$, $Arg^{3,4}$, Tyr^6)-VIP (3 μM)] for 20 min, CA secretory responses evoked by ACh, high potassium, DMPP, McN-A-343, Bay-K-8644 and cyclopiazonic acid were not altered greatly in comparison to the case of PACAP-treatment only. Taken together, these results suggest that PACAP causes the marked inhibition of CA secretion evoked by stimulation of cholinergic (both nicotinic and muscarinic) receptors as well as by membrane depolarization, indicating that this effect may be mediated by inhibiting influx of extracellular calcium and release in intracellular calcium in the rat adrenomedullary chromaffin cells.

Key Words: PACAP, Adrenal gland, Catecholamine secretion

INTRODUCTION

Pituitary adenylate cyclase-activating polypeptides (PACAP), with either 38- or 27-amino acid residues, are new members of the secretin/glucagon/vasoactive intestinal peptide (VIP) family (Arimura, 1992; Christophe, 1993; Rawlings & Hezareh, 1996). These peptides were originally isolated from ovine hypothalami and characterized by their ability to stimulate accumulation of cyclic AMP in the rat anterior pituitary cells. It has been also demonstrated that binding sites for PACAP are not limited to the brain but are widely distributed in different peripheral tissues, testis, adrenal gland, lung, liver, stomach, duodenum, jejunum, etc. (Arimura et al, 1991). PACAP shows a 68% sequence homology with VIP in the N-terminal 28 residues (Miyata et al, 1989) and binds to membranes from several tissues including rat adrenal, testis, and hypothalamus with much higher potencies than VIP. These studies indicate the existence of a more selective type of receptor for PACAP in these tissues (Gottschall et al, 1990). Adrenal gland has been reported to contain the second highest concentration of PACAP among the rat organs (Arimura et al, 1990). Recently, it has shown that PACAP activates voltage-dependent Ca^{2+} channels and phospholipase C as well as adenylate cyclase in cultured porcine adrenal medullary cells, and strongly suggest that PACAP-induced catecholamine (CA) secretion is mainly mediated by activation of voltage-dependent Ca^{2+} channels (Isobe et al, 1993).

Corresponding to: Dong-Yoon Lim, Department of Pharmacology, College of Medicine, Chosun University, 375 Seosuk-dong, Dong-gu, Kwangju 501-759, Korea. (Tel) 062-220-3659, (Fax) 062-227-4693, (E-mail) dylim@mail.chosun.ac.kr

More recently, it has been reported that PACAP forms a positive feedback loop to stimulate both rapid Ca^{2+} release from ryanodine-sensitive Ca^{2+} stores and long-lasting Ca^{2+} entry via L-type Ca^{2+} channels in bovine adrenal chromaffin cells (Tanaka et al, 1997). PACAP has been shown to relax isolated rabbit aortic smooth muscle and decrease systemic arterial pressure (AP) in the rat in a manner similar to that produced by VIP (Nandha et al, 1991; Warren et al, 1991). It has been also suggested that the hypotensive effects of PACAP and VIP in the rat are mediated by the same receptor; however, a study in the cat has shown that PACAP and VIP produce a different pattern of cardiovascular responses in the pulmonary and systemic vascular beds (Nandha et al, 1991; Minkes et al, 1992). Moreover, recent data suggest that responses to PACAP and VIP are mediated by distinct receptors and that pressor responses to PACAP are due to the release of catecholamines from the adrenal gland (Minkes et al, 1992).

In the present study, it was attempted to investigate the effect of PACAP on the CA secretory responses evoked by stimulation of cholinergic receptors and membrane depolarization in the isolated perfused rat adrenal glands, and to establish its mechanism of the action.

METHODS

Experimental procedure

Male Sprague-Dawley rats, weighing 180 to 300 grams, were anesthetized with thiopental sodium (40 mg/kg) intraperitoneally. The adrenal gland was isolated by the methods described previously (Wakade, 1981). The abdomen was opened by a midline incision, and the left adrenal gland and surrounding area were exposed by placing three hook retractors. The stomach, intestine and portion of the liver were not removed, but pushed over to the right side and covered by saline-soaked gauge pads and urine in bladder was removed in order to obtain enough working space for tying blood vessels and cannulations.

A cannula, used for perfusion of the adrenal gland, was inserted into the distal end of the renal vein after all branches of adrenal vein (if any), vena cava and aorta were ligated. Heparin (400 IU/ml) was injected into vena cava to prevent blood coagulation before ligating vessels and cannulations. A small slit was made into the adrenal cortex just opposite entrance of adrenal vein. Perfusion of the gland was started, making sure that no leakage was present, and the perfusion fluid escaped only from the slit made in adrenal cortex. Then the adrenal gland, along with ligated blood vessels and the cannula was carefully removed from the animal and placed on a platform of a leucite chamber. The chamber was continuously circulated with water heated at $37 \pm 1^{\circ}C$.

Perfusion of adrenal gland

The adrenal glands were perfused by means of a ISCO pump (WIZ Co.) at a rate of 0.3 ml/min. The perfusion was carried out with Krebs-bicarbonate solution of following composition (mM): NaCl, 118.4; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.18; $NaHCO_3$, 25; KH_2PO_4, 1.2; glucose, 11.7. The solution was constantly bubbled with 95% O_2+5% CO_2 and the final pH of the solution was maintained at 7.4 ± 0.05. The solution contained disodium EDTA (10 μg/ml) and ascorbic acid (100 ug/ml) to prevent oxidation of catecholamine.

Drug administration

The perfusions of DMPP (100 μM) and McN-A-343 (100 μM) for 2 minutes and/or a single injection of ACh (5.32 mM) and KCl (56 mM) in a volume of 0.05 ml were made into perfusion stream via a three way stopcock, and Bay-K-8644 (10^{-5} M) and cyclopiazonic acid (10^{-5} M) were also perfused for 4 min, respectively.

In the preliminary experiments it was found that upon administration of the above drugs, secretory responses to ACh, KCl, McN-A-343, Bay-K-8644 and cyclopiazonic acid returned to preinjection level in about 4 min, but the responses to DMPP in 8 min.

Collection of perfusate

As a rule, prior to stimulation with various secretagogues, perfusate was collected for 4 min to determine the spontaneous secretion of CA (background sample). Immediately after the collection of the background sample, collection of the perfusates was continued in another tube as soon as the perfusion medium containing the stimulatory agent reached the adrenal gland. Stimulated sample's was collected for 4 to 8 min. The amounts secreted in the

background sample have been subtracted from that secrected from the stimulated sample to obtain the net secretion value of CA, which is shown in all of the figures.

To study effects of PACAP on the spontaneous and evoked secretion, the adrenal gland was perfused with Krebs solution containing a potassium channel opener for 20 min, then the perfusate was collected for a certain (background sample), and then the medium was changed to the one containing the stimulating agent and the perfusates were collected for the same period as that for the background sample. Generally, the adrenal gland's perfusate was collected in chilled tubes.

Measurement of catecholamines

CA content of perfusate was measured directly by the fluorometric method of Anton & Sayre (1962) without the intermediate purification alumina for the reasons described earlier (Wakade, 1981) using fluorospectrophotometer (Kontron Co. Italy).

A volume of 0.2 ml of the perfusate was used for the reaction. The CA content in the perfusate of stimulated glands by secretagogues used in the present work was high enough to obtain readings several folds greater than the reading of control samples (unstimulated). The sample blanks were also lowest for perfusates of stimulated and non-stimulated samples. The content of CA in the perfusate was expressed in terms of norepinephrine (base) equivalents.

Statistical analysis

Statistical significance between groups was examined by Student's t-test. A P-value of less than 0.05 was considered to represent statistically significant changes unless specifically noted in the text. Values given in the text refer to means and the standard errors of the mean (S.E.M.).

The statistical analysis of the experimental results was made by computer program described by Tallarida & Murray (1987).

Drugs and their sources

The following drugs were used: Pituitary adenylate cyclase-activating polypeptide, acetylcholine chloride, 1.1-dimethyl-4-phenyl piperazinium iodide (DMPP), (Lys^1, $Pro^{2,5}$, $Arg^{3,4}$, Tyr^6)-vasoactive intestinal polypeptide, norepinephrine bitartrate, methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-arboxylate (BAY-K-8644) (Sigma Chemical Co., U.S.A.), neuropeptide Y, cyclopiazonic acid, (3-(m-cholro-phenyl-carbamoyl-oxy)-2-butynyl trimethyl ammonium chloride [McN-A-343] (RBI, U.S.A.). Drugs were dissolved in distilled water (stock) and added to the normal Krebs solution as required except Bay-K-8644, which was dissolved in 99.5% ethanol and diluted appropriately (final concentration of alcohol was less than 0.1%). Concentrations of all drugs used are expressed in terms of molar base.

RESULTS

Effect of pituitary adenylate-cyclase activating polypeptide (PACAP) on CA secretion evoked by ACh and high K^+ from the perfused rat adrenal glands

After the initial perfusion with oxygenated Krebs-bicarbonate solution for 1 hr, basal CA release from the isolated perfused rat adrenal glands amounted to 18.6 ± 2.2 ng/2 min (n=6). In the present study, PACAP itself did not produce any effect on basal CA output from perfused rat adrenal glands (data not shown). Therefore, it was decided to investigate the effects of PACAP on cholinergic receptor stimulation- as well as membrane depolarization-mediated CA secretion.

When ACh (5.32 mM) in a volume of 0.05 ml was injected into the perfusion stream, the amounts of CA secreted was 582 ± 66 ng for 4 min. However, Under the presence of 10 nM PACAP, ACh-stimulated CA secretion was significantly decreased to 402 ± 51 ng (15~19 min, $P<0.05$), 150 ± 30 ng (30~34 min, $P<0.01$), 105 ± 12 ng (45~49 min, $P<0.01$) and 138 ± 21 ng (60~64 min, $P<0.01$) from 7 adrenal glands as shown in Fig. 1.

Also, it is found that KCl, a depolarizing agent, stimulates sharply CA secretion. In the present work, excess K^+ (56 mM)-stimulated CA secretion after the pretreatment with 10 nM PACAP was markedly inhibited. In the presence of 10 nM PACAP, it amounted to 192 ± 30 ng (15~19 min, $P<0.01$), 153 ± 39 ng (30~34 min, $P<0.01$), 168 ± 33 ng (45~49 min, $P<0.01$) and 132 ± 36 ng (60~64 min, $P<0.01$) as compared with its corresponding control secretion of 348 ± 69 ng from 9 glands (Fig. 1).

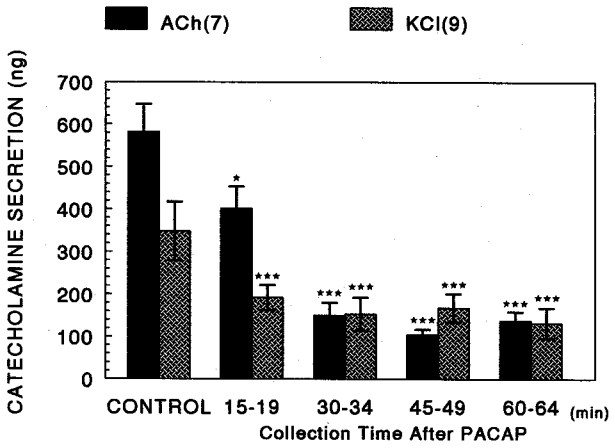

Fig. 1. Influence of pituitary adenylate cyclase-activating polypeptide (PACAP) on ACh- and high K^+-stimulated catecholamine secretion from the isolated perfused rat adrenal glands. CA secretion was induced by a single injection of ACh (5.32×10^{-3} M) or high K^+ (5.6×10^{-2} M) after perfusion with normal Krebs solution for one hour before initiation of the experimental protocol. "CONTROL" denotes CA secretion evoked by ACh or high K^+, before PACAP and then followed by treatment with 10 nM PACAP, respectively. Number in the parenthesis indicates number of experimental rat adrenal glands. Vertical bars represent the standard error of the mean (S.E.M.). Statistical difference was obtained by comparing the control with the PACAP-treated group. Each perfusate was collected for 4 minutes. ACh: acetylcholine. *: $P<0.05$, ***: $P<0.01$

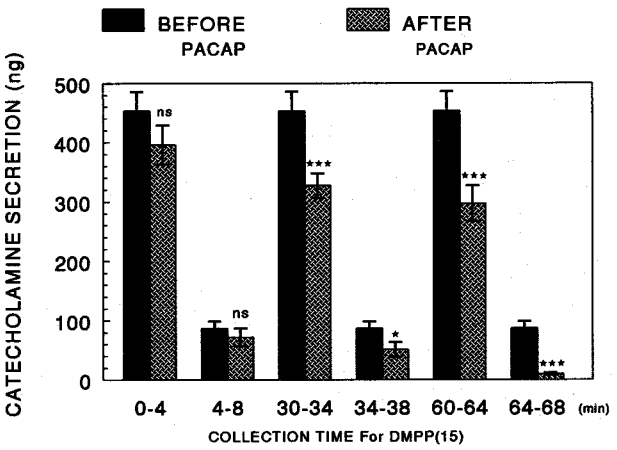

Fig. 2. Influence of PACAP on DMPP-stimulated catecholamine secretion from the isolated perfused rat adrenal glands. "BEFORE" and "AFTER" denote CA secretion evoked by DMPP (10^{-4} M) before and after treatment with 10 nM PACAP, respectively. Pefusate for DMPP was collected for 8 min at intervals of 4 min. Other legends are the same as in Fig. 1. DMPP: 1,1-dimethy-4-phenyl pieprazinium. *: $P<0.01$, ns: Nonsignificant.

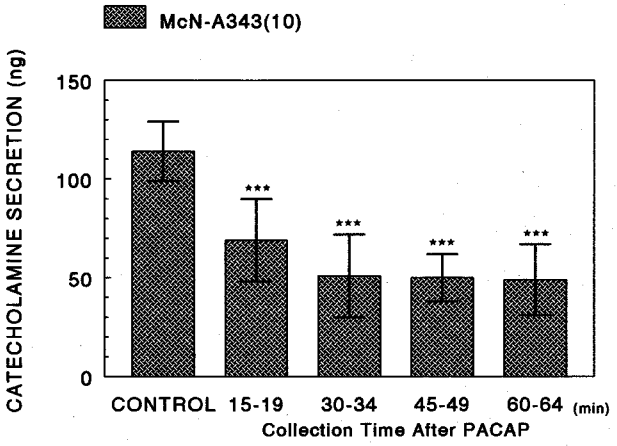

Fig. 3. Influence of PACAP on McN-A-343-stimulated catecholamine secretion from the isolated perfused rat adrenal glands. McN-A-343 (10^{-4} M) was given before and after treatment with 10 nM PACAP, respectively. Other legends are the same as in Fig. 1. McN-A-343: (3-(m-cholro-phenyl-carbamoyl-oxy)-2-butynyl trimethyl ammonium chloride. ***: $P<0.01$.

Effect of pituitary adenylate-cyclase activating polypeptide on CA secretion evoked by DMPP and McN-A-343 from the perfused rat adrenal glands

When perfused through the rat adrenal gland, DMPP (100 μM for 2 min), which is a selective nicotinic receptor agonist in autonomic sympathetic ganglia, evoked a sharp and rapid increase in CA secretion. As shown in Fig. 2, DMPP-stimulated CA secretion before preloading with 10 nM PACAP was 453 ± 33 ng (0~4 min) and 87 ± 12 ng (4~8 min), while after pretreatment with 10 nM PACAP they were reduced to 396 ± 207 ng (0~4 min, ns) and 72 ± 15 ng (4~8 min, ns), 327 ± 21 ng (30~34 min, $P<0.01$) and 51 ± 12 ng (34~38 min, $P<0.05$), 297 ± 30 ng (60~64 min, $P<0.01$) and 10 ± 2 ng (64~68 min, $P<0.01$), respectively from 15 rat adrenal glands.

As illustrated in Fig. 3, McN-A-343 (100 μM), which is a selective muscarinic M_1- agonist (Hammer & Giachetti, 1982), perfused into an adrenal gland for 4 min caused an increased CA secretion to 114 ± 15 ng for 4 min from 10 experiments. However, McN-A-343-stimulated CA secretion in the presence of 10

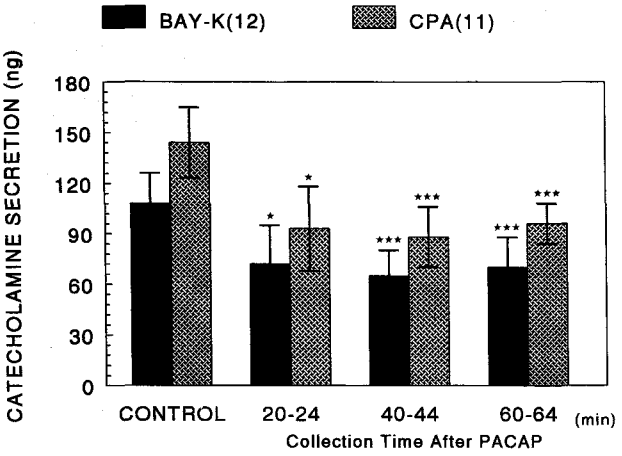

Fig. 4. Influence of PACAP on Bay-K-8644- and CPA-stimulated catecholamine secretion from the isolated perfused rat adrenal glands. Bay-K-8644 (10^{-5} M) and CPA (10^{-5} M) were given before and after treatment with 10 nM PACAP, respectively Other legends are the same as in Fig. 1. CPA: Cyclopiazonic acid. *: $P<0.05$, ***: $P<0.01$

nM PACAP was markedly inhibited to 69±21 ng (15~19 min, $P<0.01$), 51±21 ng (30~34 min, $P<0.01$), 50±12 ng (45~49 min, $P<0.01$) and 49±18 ng (60~64 min, $P<0.01$) min, which were 61% (15~19 min), 45% (30~34 min), 44% (45~49 min) and 43% (60~64 min) of the corresponding control secretion, respectively.

Effect of PACAP on CA secretion evoked by Bay-K-8644 and cyclopiazonic acid from the perfused rat adrenal glands

Since Bay-K-8644 is known to be a calcium channel activator which causes positive inotropy and vasoconstriction in isolated tissues and intact animals (Schramm et al, 1982; Wada et al, 1985) and enhances basal Ca^{2+} uptake (Garcia et al, 1984) and CA release (Lim et al, 1992), it was of interest to determine the effects of 10 nM PACAP on Bay-K-8644-stimulated CA secretion from the isolated perfused rat adrenal glands. Fig. 4 illustrates the inhibitory effect of 10 nM PACAP on Bay-K-8644- and cyclopiazonic acid-evoked CA secretory responses. Bay-K-8644 (10 μM) given into the perfusion stream for 4 min increased CA secretion to 108±18 ng/4 min from 12 rat adrenal glands. However, under the effect of 1 nM PACAP which was preloaded for 20 min before Bay-K-8644 was introduced, Bay-K-8644 -stimulated CA secretion was strikingly depressed to 72±23 ng (20~24 min, $P<0.05$), 65±15 ng (40~44 min, $P<0.01$), and 70±18 ng (60~64 min, $P<0.01$) min, respectively, as compared to the corresponding control release; thus, the releasing responses were reduced to 67% (20~24 min), 57% (40~44 min), and 65% (60~64 min) of the corresponding control response, respectively.

Cyclopiazonic acid, a mycotoxin from *Aspergillus* and *Penicillium*, has been described as a highly selective inhibitor of Ca^{2+}-ATPase in skeletal muscle sarcoplasmic reticulum (Georger & Riley, 1989; Seidler et al, 1989). It may be extremely valuable pharmacological tool for investigating intracellular Ca^{2+} mobilization and ionic current regulated by intracellular calcium (Suzuki et al, 1992).

The inhibitory action of PACAP on cyclopiazonic acid-evoked CA secretory response was observed as shown in Fig. 4. Under the effect of 10 nM PACAP in 11 rat adrenal glands, cyclopiazonic acid (10 M)-evoked CA secretion was reduced to 93 ± 25 ng (20~24 min, $P<0.05$), 88±18 ng (40~44 min, $P<0.01$), and 96±12 ng (60~64 min, $P<0.01$) min, respectively, as compared with 144±21 ng for 4 min of control response.

The effects of PACAP plus VIP-antagonist on CA release evoked by ACh, excess K^+, DMPP, McN-A-343, Bay-K-8644 and cyclopiazonic acid from the perfused rat adrenal glands

Since it has been found that PACAP shows a 68% sequence homology with VIP in the N-terminal 28 residues (Miyata et al, 1989) and relaxes isolated rabbit aortic smooth muscle and decreases systemic arterial pressure (AP) in the rat in a manner similar to that produced by VIP (Nandha et al, 1991; Warren et al, 1991), it was tried to determine the effect of PACAP in the presence of VIP-antagonist on CA secretion evoked by various secretagogues from the isolated rat adrenal glands.

ACh (5.32 mM)- and excess K^+ (56 mM)-induced CA releases in the presence of 10 nM PACAP along with 3 μM VIP-antagonist for 20 min amounted to 52±9% (ns, n=8) and 53±7% (ns, n=12), respectively as compared to their secretory responses of 50±10% and 48±10% of their controls in the presence of 10 nM PACAP only (Fig. 5).

On the other hand, PACAP in the presence of VIP-antagonist treatment did fail to alter the basal

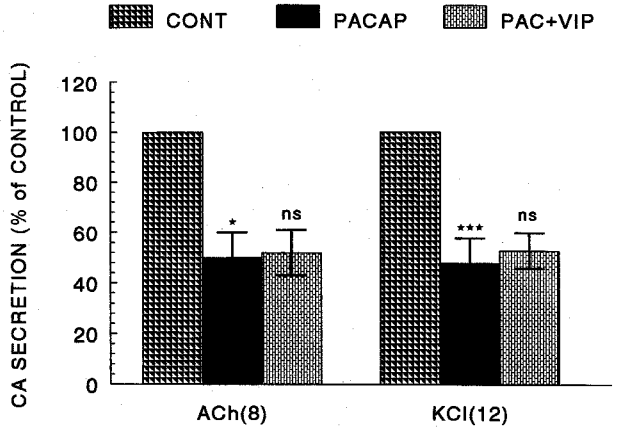

Fig. 5. Effect of PACAP in the presence of VIP antagonist on ACh- and high K^+-evoked catecholamine (CA) release. ACh (5.32×10^{-3} M) and high K^+ (5.6×10^{-2} M) were induced before and after preloading with PACAP (10^{-8} M) plus (Lys[1], Pro[2.5], Arg[3.4], Tyr[6])-VIP (10^{-6} M) 1 M for 20 min, respectively. Other legends are the same as in Fig. 1. CONT: Control, PACAP: pituitary adenylate cyclase-activating polypeptide. PACAP+VIP: PACAP+VIP antagonist [(Lys[1], Pro[2.5], Arg[3.4], Tyr[6])-VIP]. *: $P<0.05$, ***: $P<0.01$. ns: Statistically nonsignificant.

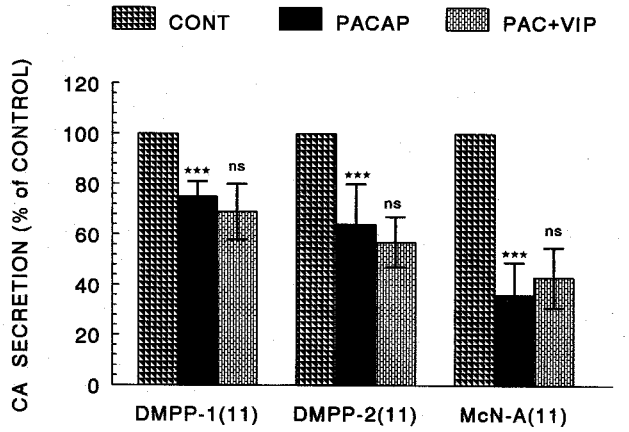

Fig. 6. Effect of PACAP in the presence of VIP antagonist on DMPP- and McN-A-343-evoked CA release. DMPP (10^{-4} M) and McN-A-343 (10^{-4} M) were administered before and after pretreatment with Krebs solution containing 10 nM PACAP plus 3 μM VIP-antagonist for 20 min, respectively. Other legends are the same as in Fig. 1 and 5. ***: $P<0.01$, ns: Nonsignificant.

CA secretory response (data not shown). As depicted in Fig. 6, DMPP (100 μM)- and McN-A-343 (100 μM)-induced CA secretions under the existence of

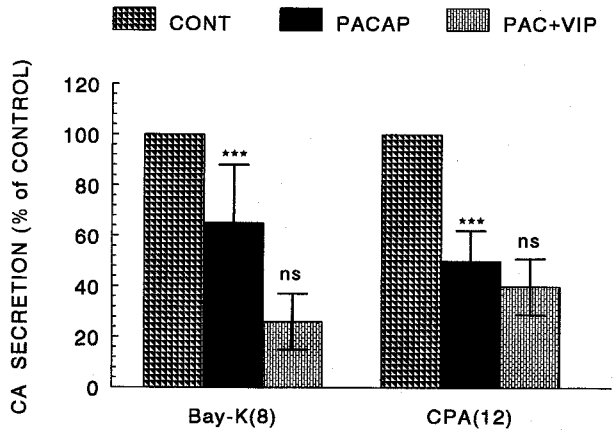

Fig. 7. Effect of PACAP plus VIP-antagonist on Bay-K-8644- and CPA-evoked CA release. Bay-K-8644 (10^{-5} M) and CPA (10^{-5} M) were administered before and after pretreatment with Krebs solution containing 10 nM PACAP plus 3 μM VIP-antagonist for 20 min, respectively. Other legends are the same as in Fig. 1 and 5. *: $P<0.05$, ***: $P<0.01$, ns: Nonsignificant. CPA: Cyclopiazonic acid.

PACAP plus VIP-antagonist were $69\pm11\%$ (0~4 min, ns, n=11) and $57\pm11\%$ (4~8 min, ns, n=11), and $43\pm12\%$ (0~4 min, ns, n=11) of their corresponding control responses, respectively as compared to the secretory responses of $75\pm6\%$ (0~4 min) and $64\pm16\%$ (4~8 min), and 36 ± 13 (0~4 min) of the control in the presence of 10 nM PACAP only.

CPA (10 μM)- and Bay-K-8644 (10 μM)-induced CA secretory responses after preloading with Krebs solution containing 10 nM PACAP along with 3 μM VIP-antagonist for 20 min amounted to $56\pm11\%$ (ns, n=8) and $40\pm11\%$ (ns, n=12), respectively, as compared to $50\pm11\%$ and $47\pm10\%$ of each CA secretory response in the presence of PACAP only as shown in Fig. 7.

Effect of neuropeptide Y on CA secretion evoked by ACh, excess K^+, DMPP, McN-A-343, Bay-K-8644 and cyclopiazonic acid from the perfused rat adrenal glands

In the experimental results as shown in Fig. 1-7, it was found that PACAP showed a great inhibition in various secretagogues-induced CA secretory responses. Since it has been known that neuropeptide Y is co-localized with norepinephrine in peripheral sympathetic nerves (Lundberg et al, 1990) and inhibits the nicotine-mediated CA release from bovine

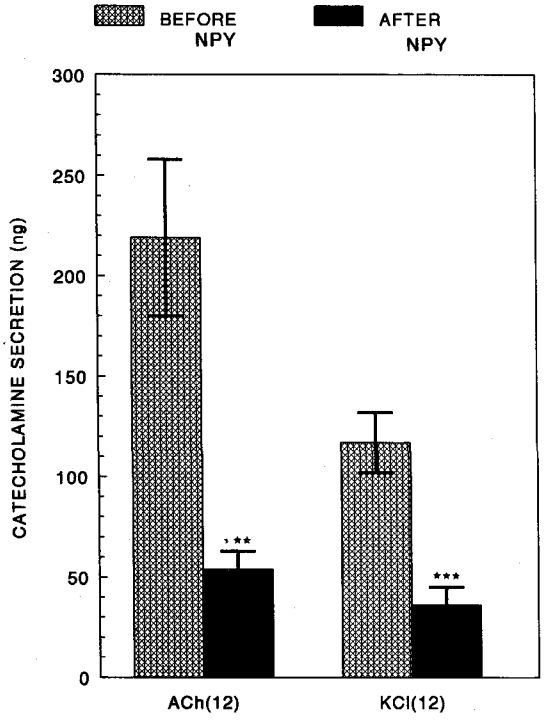

Fig. 8. Effect of neuropeptide Y on catecholamine release evoked by ACh and high K^+. ACh (5.32×10^{-3} M) and high K^+ (5.6×10^{-2} M) were administered before and after pretreatment with 2 μM neuropeptide Y for 20 min, respectively. Other legends are the same as in Fig. 1. NPY: Neuropeptide Y. Ach: Acetylcholine. ***: $P<0.01$

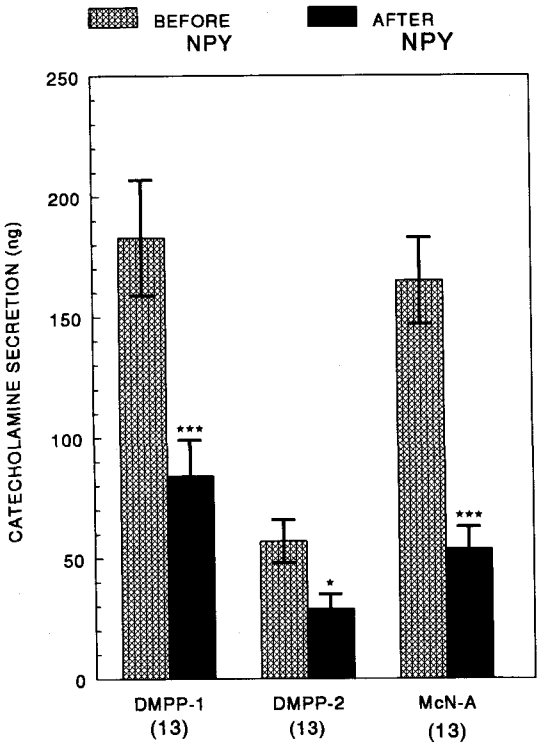

Fig. 9. Influence of neuropeptide Y on DMPP- and McN-A-343 (McN-A)-stimulated catecholamine secretory responses. DMPP (10^{-4} M) and McN-A-343 (10^{-4} M) were administered before and after pretreatment with 2 μM neuropeptide Y for 20 min, respectively. Other legends are the same as in Fig. 1. NPY: Neuropeptide. *: $P<0.05$, ***: $P<0.01$.

adrenal chromaffin cells (Higuchi et al, 1988), it is likely of very interest to examine the effect of neuropeptide Y on CA secretion evoked by various secretagogues.

CA releasing responses evoked by ACh (5.32 mM) and excess K^+ (56 mM) after preloading with 2 M neuropeptide Y for 20 min were greatly inhibited to 54 ± 9 ng ($P<0.01$, n=12) and 36 ± 9 ng ($P<0.01$, n=12) for 4 min, respectively as compared to each corresponding control secretion of 219 ± 39 ng and 117 ± 15 ng for 4 min as shown in Fig. 8.

DMPP (100 μM)- and McN-A-343 (100 μM)-stimulated CA releases in the absence of neuropeptide Y were 183 ± 24 ng (0~4 min) and 57 ± 9 ng (4~8 min), and 165 ± 18 ng (0~4 min), respectively. However, after preloading with 2 μM neuropeptide Y for 20 min, they were significantly reduced to 84 ± 15 ng (0~4 min, $P<0.01$, n=13) and 29 ± 6 ng (4~8 min, $P<0.05$, n=13), and 54 ± 9 ng (0~4 min, $P<0.01$, n=13), respectively, as compared to their corresponding control responses. Fig. 9 illustrates that neuropeptide Y inhibits markedly CA release evoked by DMPP and McN-A-343.

As illustrated in Fig. 10, the inhibitory effect of neuropeptide Y on Bay-K-8644-evoked CA secretion was observed. Bay-K-8644 (10 μM) given into the perfusion stream for 4 min increased CA secretion to 144 ± 21 ng from 12 rat adrenal glands. However, under the effect of 2 μM neuropeptide Y which was preloaded 20 min before the perfusion of Bay-K-8644, Bay-K-8644-stimulated CA secretion was strikingly depressed to 57 ± 12 ng ($P<0.01$) for 4 min as compared to the corresponding control release; thus, the release was reduced to 40% of the control secretion.

In the presence of 2 μM neuropeptide Y, cyclopiazonic acid (10 μM) given into the adrenal gland for 4 min caused the CA secretory response of 51 ± 6 ng ($P<0.01$) as compared to the corresponding control response of 132 ± 18 ng from 12 experiments, as

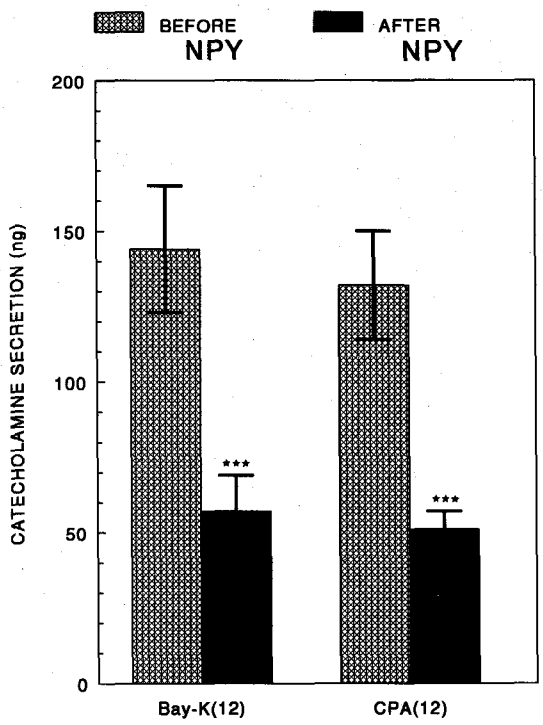

Fig. 10. Influence of neuropeptide Y on Bay-K-8644- and CPA-stimulated catecholamine secretory responses. Bay-K-8644 (10^{-5} M) and CPA (10^{-5} M) were administered before and after pretreatment with 2 μM neuropeptide Y for 20 min, respectively. Other legends are the same as in Fig. 1. NPY: Neuropeptide Y. CPA: Cyclopiazonic acid. ***: $P<0.01$

shown in Fig. 10.

DISCUSSION

The present experimental results demonstrate that PACAP causes significant inhibition of CA secretory responses evoked by stimulation of cholinergic (both nicotinic and muscarinic) receptors as well as by membrane depolarization, indicating that this effect may be mediated by inhibiting influx of extracellular calcium and release in intracellular calcium in the rat adrenomedullary chromaffin cells.

However, the present experimental results are not in agreement with the previous report (Isobe et al, 1993), in which PACAP is found to activate voltage-dependent Ca^{2+} channels and phospholipase C as well as adenylate cyclase in culture porcine adrenal medullary cells and to induce CA secretion mainly by activation of voltage-dependent Ca^{2+} channels. In the present study in the isolated perfused rat adrenal gland, PACAP produced decreases on cholinergic stimulation- and membrane depolarization-induced CA secretory responses. The reason for differences in results of the present study and previous reports in cultured porcine adrenal medullary chromaffin cells is uncertain, but may be related to species differences or differences in experimental design (Nandha et al, 1991; Warren et al, 1991; Isobe et al, 1993). In support of this idea, PACAP and VIP have been shown to produce similar decreases in arterial pressure in the rat and relax isolated rabbit aortic smooth muscle. It has been suggested that the vasodilatation produced by PACAP and VIP in the rat is mediated by the same receptor (Nandha et al, 1991; Warren et al, 1991). However, Isobe and his coworkers (1992) have shown that PACAP produces decreases and biphasic changes in arterial pressure and systemic vascular resistance, whereas VIP produces only decreases in arterial pressure and systemic vascular resistance. Moreover, PACAP produced increases in pulmonary vascular resistance, whereas VIP produced little or no effect on pulmonary vascular resistance. Therefore, it was suggest that the reason for the differences in the vascular responses to PACAP and VIP is due to species differences or differences in experimental design (Nandha et al, 1991; Warren et al, 1991).

In the present study, the findings that PACAP inhibits CA secretory responses evoked by DMPP (a nicotinic receptor agonist), McN-A-343 (a muscaric M_1-receptor agonist) and high potassium (membrane depolarization) in the isolated perfused rat adrenal gland suggest that this inhibitory effect of PACAP is not due to the increased cAMP. However, PACAP was demonstrated to activate adenylate cylcase in PC-12 cells and several other cell types (Myata et al, 1989; Watanabe et al, 1990).

In support of this finding, Isobe and his coworkers (1993) have demonstrated that the cAMP messenger system dose not mediate the immediate effect of PACAP on CA secretion in cultured porcine adrenal medullary cells. In their studies, firstly, forskolin, an activator of adenylate cyclase, when added either alone or in combination with high K^+, which stimulates Ca^{2+} influx and/or a phorbor ester, which activates the protein kinase C, does not induce or potentiate the CA secretion. Secondly, the addition of IBMX, which induced an increase in cellular cAMP content by inhibiting cyclic nucleotide phosphodiesterase, has no enhancing effect on the PACAP-

induced CA secretion. Thirdly, removal of extracellular Ca^{2+} enhances the PACAP-induced increase in cAMP content, but does abolish the PACAP-induced CA release. Fourthly, the addition of Rp-cyclic AMPS, a nonhydrolyzable competitive inhibitor for the cAMP-dependent protein kinase, dose not have any inhibitory effect on the PACAP-induced response. However, cAMP may have some permissive long-term effects on the CA production because cAMP was reported to stimulate the expression of tyrosine hydroxylase messenger RNA, a rate-limiting enzyme in the CA synthesis in adrenal medullary cells (Meligeni et al, 1982; Lewis et al, 1987). In general, PACAP 27 exhibits 68% homology with VIP, and the 10 amino-terminal residues of PACAP and 60% homologous to growth hormone-releasing hormone (Miyata et al, 1989). Although the physiological activities of PACAP and VIP are similar in some tissues, PACAP is ~1000-fold more potent than VIP in many neuroendocrine systems (Miyata et al, 1989; Deutsch & Sun, 1992). The diverse activities of PACAP have been postulated to be mediated by multiple target tissue receptors (Shivers et al, 1991: Ishihara et al, 1992; Morrow et al, 1993; Spengler et al, 1993; Hamar & Lutz, 1994). The type I PACAP receptor exhibits high affinity for both PACAP peptides but binds VIP with 1,000-fold lower affinity. The type II PACAP receptor binds PACAPs and VIP with apparent equal affinity and may be the classical VIP receptor (Ishihara et al, 1992; Spengler et al, 1993). However, in the present investigation, the result that PACAP-induced inhibitory effect on CA secretory responses evoked by DMPP and McN-A-343 as well as high K^+ was not affected by co-treatment with VIP antagonist indicates that this inhibitory effect of PACAP is not associated with VIP receptors. This finding is not consistent with previous results that hypotensive effects of PACAP and VIP in the rat are mediated by the same receptors; however, another study in the cat has shown that PACAP and VIP produce a different pattern of cardiovascular responses in the pulmonary and systemic vascular beds (Nandha et al, 1991; Minkes et al, 1992).

In this study, neuropeptide Y also produced a great decrease in CA secretory responses evoked by cholinergic stimulation and membrane depolarization. Neuropeptide Y, a 36-amino acid residue peptide originally isolated from porcine brain is co-localized with norepinephrine in peripheral sympathetic nerves (Lundberg et al, 1990). It has been also identified in the adrenal medulla of various animal species including dogs (Allen et al, 1983; Majane et al, 1985). Moreover, PACAP was also found to stimulate neuropeptide Y and CA secretion in primary cultured superior cervical ganglion neuron (May & Braas, 1995). In the present study, although it could not be demonstrated whether neuropeptide Y receptor is located adrenal chromaffin cells or not, neuropeptide Y inhibited markedly the CA secretory effects evoked by DMPP and McN-A-343 as well as membrane depolarization.

In support of this result, it has been shown that neuropeptide Y inhibits the nicotine-mediated release of CA from bovine adrenal chromaffin cells (Higuchi et al, 1988). Generally, nicotine, a nicotinic cholinergic agonist, which activated voltage-dependent Ca^{2+} channels via the induction of the membrane depolarization, is also very potent secretagogue for adrenal medullary cells (Holz et al, 1982).

In the present investigation, the results that PACAP as well as neuropeptide Y inhibits CA secretion evoked by stimulation of nicotinic and muscarinic receptors with DMPP and McN-A-343 suggest that PACAP receptors are involved in the regulation of the overall secretory responses evoked by nicotinic and muscarinic stimulation. In support of this hypothesis, it has been shown that muscarinic stimulation generates a depolarizing signal which triggers the firing of action potentials, resulting in the increased CA release in the rat chromaffin cells (Akaike et al, 1990; Lim & Hwang, 1991). These observations are in line with a previous report (Ladona et al, 1987; Uceda et al, 1992) showing that Bay-K-8644 almost tripled the peak secretory response to muscarine in perfused cat adrenal glands. In this experiment, both PACAP also depress greatly CA secretion induced by Bay-K-8644, which is found to potentiate the release of CA by increasing Ca^{2+} influx through L-type Ca^{2+} channels in chromaffin cells (Garcia et al, 1984). These findings that PACAP inhibited CA secretion evoked by high K^+ and also by Bay-K-8644 suggest that this PACAP inhibits directly the voltage-dependent Ca^{2+} channels through PACAP receptors, just like Ca^{2+} channel blockers (Cena et al, 1983), which have direct actions on voltage-dependent Ca^{2+} channels. In the bovine chromaffin cells, stimulation of nicotinic, but not muscarinic ACh receptors is known to cause CA secretion by increasing Ca^{2+} influx largely through voltage-dependent Ca^{2+} chan-

nels (Oka et al, 1979; Burgoyne, 1984). Therefore, it seems that the PACAP inhibits CA secretion evoked by DMPP by inhibiting Ca^{2+} influx through voltage-dependent Ca^{2+} channels activated by nicotinic ACh receptors with DMPP.

It has been also shown that PACAP inhibits the increase in CA secretion evoked by cyclopiazonic acid, which is known to be a highly selective inhibitor of Ca^{2+}-ATPase in skeletal muscle sarcoplasmic reticulum (Geoger & Riley, 1989; Seidler et al, 1989) and a valuable pharmacological tool for investigating intracellular Ca^{2+} mobilization and ionic currents regulated by intracellular Ca^{2+} (Suzuki et al, 1992). Therefore, it is felt that the inhibitory effect of PACAP on CA secretion evoked by cholinergic stimulation as well as by membrane-depolarization may be associated with the mobilization of intracellular Ca^{2+} in the chromaffin cells. This indicates that the PACAP has an inhibitory effect on the release of Ca^{2+} from the intracellular pools induced by stimulation of muscarinic ACh receptors, which is weakly responsible for the secretion of CA. It has been shown that Ca^{2+}-uptake into intracellular storage sites susceptible to caffeine (Ilno, 1989) is almost completely abolished by treatment with cyclopiazonic acid during the proceeding Ca^{2+} load (Suzuki et al, 1992). This is consistent with the findings obtained in skinned smooth muscle fibers of the longitudinal layer of the guinea-pig ileum, where Ca^{2+}-uptake was also inhibited by cyclopiazonic acid (Uyama et al, 1992). Suzuki and his coworkers (1992) have shown that cyclopiazonic acid easily penetrates into the cytoplasm through the plasma membrane and reduces Ca^{2+}-ATPase activity in sarcoplasmic/endoplasmic reticulum, resulting in increase in the subsequent Ca^{2+} release from those storage sites. Moreover, in bovine adrenal chromaffin cells, stimulation of muscarinic ACh receptors is also proposed to cause activation of phosphoinositide metabolism, resulting in the formation of inositol 1, 4, 5-trisphosphate, which induces the mobilization of Ca^{2+} from the intracellular pools (Cheek et al, 1989; Challis et al, 1991). However, in the present study, it is uncertain whether the inhibitory effect of the PACAP on Ca^{2+} movement from intracellular pools is due to their direct effect on the PI response or an indirect effect as a result of the membrane hyperpolarization induced by PACAP receptors.

In the present study, the fact that McN-A-343-evoked CA secretion was depressed by pretreatment with PACAP appears to be consistent with these previous results. Furthermore, in the absence of extracellular Ca^{2+}, methacholine still evoked a transient Ca^{2+} rise that declined quickly to basal levels, suggesting the release of Ca^{2+} from an intracellular pool likely associated with the smooth endoplasmic reticulum in cat chromaffin cells (Uceda et al, 1994). This observation is in line with facts that muscarinic stimulation of bovine chromaffin cells increases the formation of inositol trisphosphate (Forsberg et al, 1986) and that inositol trisphosphate mobilizes Ca^{2+} in permeabilized cells (Fohr et al, 1991). A similar rise of intracellular Ca^{2+} by muscarinic stimulation, even in the absence of extracellular Ca^{2+}, has been demonstrated in bovine chromaffin cell suspensions (Kao & Schneider, 1985, 1986; Kim & Westhead, 1989) and in cat chromaffin cells (Sorimach et al, 1992).

In conclusion, from these experimental results, it is thought that the PACAP inhibits CA secretory responses evoked by cholinergic (both nicotinic and muscarinic) stimulation as well as by membrane depolarization, resulting in inhibition of Ca^{2+} influx through the L-type voltage-dependent calcium channels and also in inhibition of Ca^{2+} mobilization from intracellular pools. There findings suggest that this PACAP may play a modulatory role in CA secretion mainly through their direct effects on the voltage-dependent membrane calcium channels.

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