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Mechanism of Vasoactive Intestinal Polypeptide-Induced Catecholamine Secretion from the Rat Adrenal Medulla

Dong-Yoon Lim, Jae-Bong Heo, Cheol-Hee Choi, Geon-Han Lim, Yong-Gyoon Lee, Song-Hoon Oh, Il-Sik Kim, and Jong-In Kim

Department of Pharmacology, College of Medicine, Chosun University, Kwangju 501–759, Korea

The present study was attempted to investigate the effect of vasoactive intestinal polypeptide (VIP) on secretion of catecholamines (CA) and to establish whether there is the existence of a noncholinergic mechanism in adrenomedullary CA secretion from the isolated perfused rat adrenal gland. The perfusion into an adrenal vein of VIP (3×10^{-6} M) for 5 min or the injection of acetylcholine (ACh, 5.32×10^{-3} M) resulted in great increases in CA secretion. Tachyphylaxis to releasing effect of CA evoked by VIP was not observed by the repeated perfusion. The net increase in adrenal CA secretion evoked by VIP still remained unaffected in the presence of atropine or chlorisondamine. However, the CA release in response to ACh was greatly inhibited by the pretreatment with atropine or chlorisondamine. The releasing effects of CA evoked by either VIP or ACh were depressed by pretreatment with nicardipine, TMB-8, and the perfusion of Ca^{2+}-free medium. Moreover, VIP- as well as ACh-evoked CA secretory responses were markedly inhibited under the presence of (Lys^{1}, $Pro^{2.5}$, $Arg^{3.4}$, Tyr^{6})-VIP or naloxone. CA secretory responses induced by ACh and high K^{+} (5.6×10^{-2} M) were potentiated by infusion of VIP (3×10^{-6}M for 5 min). Taken together, these experimental results indicate that VIP causes CA release in a fashion of calcium ion -dependence, suggesting strongly that there exists a noncholinergic mechanism that may be involved in the regulation of adrenomedullary CA secretion through VIP receptors in the rat adrenal gland, and that VIP may be the noncholinergic excitatory secretagogue present in the chromaffin cells.

Key Words: Vasoactive intestinal polypeptide, Catecholamine secretion, VIPergic receptor stimulation

INTRODUCTION

Vasoactive intestinal polypeptide is a 28-residue peptide that is widely distributed in the central and peripheral nervous systems, but it is concentrated in certain regions of the brain (cerebral cortex, hypothalamus), in the intestine, exocrine glands and in some endocrine glands. It has been also implicated in various neuronal systems as a neuropeptide co-released with classical neurotransmitters, including ACh, and may play an important role in controlling smooth muscle tone and motility, blood flow and secretion (Fahrenkrug, 1991). VIP is known to be a potent vasodilator and may mediate atropine-resistant (noncholinergic) vasodilation (Brayden & Bevan, 1986; Duckles & Said, 1987; Lee, Saito & Berezin, 1984). It was found that the stimulatory effect of exogenous ACh on the adrenal medulla of the rat was fully blocked by nicotinic and muscarinic receptor antagonists, but the secretion of catecholamines (CA) evoked by stimulation of splanchnic nerves was partially blocked by them (Malhotra & Wakade, 1987). Some studies on the isolated, retrogradely perfused, rat adrenal gland have provided evidence for a significant contribution of non-cholinergic, secretomotor nerve fibers to CA secretion elicited by a field stimulation of adrenal nerve terminals (Malhotra & Wakade, 1987a; Wakade, 1988; Wakade et al, 1991). It is likely that the main non-cholinergic trans-

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

mitter responsible for this effects is VIP (Malhotra & Wakade, 1987b; Wakade, 1991; Wakade et al, 1991) or a structurally related peptide such as a pituitary adenylate cyclase activating polypeptide (PACAP, Wakade et al, 1992).

In the rat superior cervical ganglia, it was reported that postsynaptic events triggered by stimulation presynaptic nerves were only partially blocked by cholinergic receptor antagonists. Secretin or VIP was considered to be a likely noncholinergic neurotransmitter in the superior cervical ganglia. Neuronal cell bodies and terminals containing VIP-like immunoreactivity were detected (Hokfelt et al, 1981; Holzwarth, 1984). Guo & Wakade (1994) have found the results consistent with a hypothesis that cholinergic and peptidergic transmitters control CA secretion at different levels of neuronal activity and provide new evidence that ACh stimulates the secretion of norepinephrine and epinephrine whereas VIP and PACAP predominantly stimulated the secretion of epinephrine. A recent study has also indicated that, in the isolated perfused rat adrenal gland, administration of VIP also provoked the secretion of aldosterone and corticosterone, mediated most probably via locally released epinephrine (Hinson et al, 1992). It is also reported that there exists a non-nicotinic mechanism that may be implicated in the local regulation of medullary CA secretion in the dog adrenal gland (Yamaguchi, 1993). Field stimulation of splanchnic nerves also causes the release of endogenous VIP-like immunoreactive substance (VIP-ir) from the isolated perfused rat adrenal gland (Wakade, 1991) and the anesthetized dog adrenal gland (Gaspo et al, 1995), suggesting that VIP is released along with CA from the adrenomedullary chromaffin cells. Moreover, in the isolated bovine adrenal chromaffin cells, VIP stimulates CA biosynthesis by activating tyrosine hydroxylase, suggesting that VIP is implicated in a peptidergic stimulatory mechanism of medullary CA secretion (Houch et al, 1987; Waymire et al, 1991). Numerous differences have been characterized in the pharmacology of adrenal chromaffin cells from different species. Wilson (1988) have found that VIP receptors are present on cultured bovine chromaffin cells and cause a cyclic AMP response in these cells. However, it is not a secretagogue on bovine chromaffin cells: VIP does not elicit CA secretion in the absence of nicotinic agonists (Wilson, 1988).

In the present study, it was attempted to investigate the effect of VIP on CA secretion from the perfused rat adrenal gland and to establish whether there exists VIPergic receptors related to CA secretory response.

METHODS

Experimental animals

Mature male Sprague-Dawley rats, weighing 180~300 grams, were anesthetized with ether. The adrenal gland was isolated by the methods described previously (Wakade, 1981). The abdomen was opened by a mid-line 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. Heparine (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 0.31 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 ± 0.5. The solution contained disodium EDTA (10 μg/ml) and ascorbic acid (100 μg/ml) to prevent oxidation of catecholamine.

Drug administration

Perfusion of VIP (3×10^{-6} M) for 5 min or single injection of ACh (5.32×10^{-3} M) and high K^+ (5.6×10^{-4} M) in a volume of 0.05 ml were made into perfusion stream via a three way stopcock. In the preliminary experiments it was found that upon administration of the above drugs, secretory responses to ACh and high K^+ returned to preinjection level in about 4 min and VIP within 15 min, respectively. Generally, the adrenal glands were perfused with normal Krebs solution for about one hour before the experimental protocols are initiated. The adrenal perfusate was collected in chilled tubes.

Collection of perfusate

As a rule, prior to each stimulation with VIP or ACh samples were collected (4 min) to determine the spontaneous secretion of CA ("background sample"). Immediately after the collection of the "background sample", collection of the perfusate was continued in another tube as soon as the perfusion medium containing the stimulatory agent reached the adrenal gland. Each perfusate was collected for 4 min. The amounts secreted in the "background sample" have been subtracted from those from secreted the "stimulated sample" to obtain the net secretion value of CA, which is shown in all of the figures. To study the effects of a test agent on the spontaneous and drug-evoked secretion, the adrenal gland was perfused with Krebs solution containing the agent for 20 ~30 min, then the perfusate was collected for a specific time period ("background sample"), and then the medium was changed to the one containing the test agent and the perfusate were collected for the same period as that for the "background sample".

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 (Shimadzu Co., Japan). A volume of 0.2 ml of the perfusate was used for the reaction. The CA content in the perfusate of stimulated glands by ACh or VIP was high enough to obtain readings several-fold 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. All data are presented as means with their standard errors.

Drugs and their sources

The following drugs were used: vasoactive intestinal polypeptide, acetylcholine chloride, 1. 1-dimethyl-4-phenyl piperazinium iodide (DMPP), norepinephrine bitartrate, nicardipine hydrochioride and 3.4.5-trimethoxy benzoic acid 8-(diethylamino) octylester (TMB-8), atropne sulfate and $\langle Lys^1, Pro^{2,5}, Arg^{3,4}, Tyr^6 \rangle$-vasoactive intestinal peptide from Sigma Chemical Co., U.S.A., and naloxone hydrochloride from Reyon Pharmaceutical Co., R.O.K., and chlorisondamine chloride from Ciba. Co., U.S.A., and (3-(m-chloro-phenyl-carbamoyl-oxy)-2-butynyl trimethyl ammonium chloride [McN-A-343] from RBI, U.S.A.. Drugs were dissolved in distilled water (stock) and added to the normal Krebs solution as required. Concentrations of all drugs used are expressed in terms of molar base.

Statistical analysis

The statistical significance between groups was determined by utilizing the Student's t-test. Data obtained from animals which served own an control were analyzed for the significance using t-test for paired observation. A P-value of less than 0.05 was considered to represent statistical significant changes unless specifically noted in the text. Values given in the text refer to means with standard errors of the mean (S.E.M.). The statistical analysis of the present experimental results was made by computer program of statistics described previously by Tallarida & Murray (1987).

RESULTS

The secretory effect of CA evoked by VIP

When the adrenal gland was perfused with oxygenated Krebs-bicarbonate solution for 60 min before experimental protocol is initiated, the spontaneous CA secretion reached steady state. The basal CA release from the perfused rat adrenal medulla

amounted to 24±3 ng for 2 min from 8 experiments. The releasing effects to the initial perfusion of VIP (3×10^{-6} M) are shown in Fig. 2. Administration of VIP (3×10^{-6} M) into the perfusion stream for 5 min exerted significant output of CA over the background release, which were 208±25 ng (0~5 min), 162±31 ng (5~10 min) and 102±23 ng (10~15 min). A gradual increase in VIP concentration resulted in greater amounts of CA released in the perfusate (Data not shown). These observations seem to be consistent with those reported previously in the anesthetized dog adrenals (Gaspo et al, 1995), in the isolated rat adrenal glands (Malhotra & Wakade, 1987a; Wakade, 1988; Wakade et al, 1991) and in the isolated bovine adrenal chromaffin cells (Houch et al, 1987; Waymire et al, 1991).

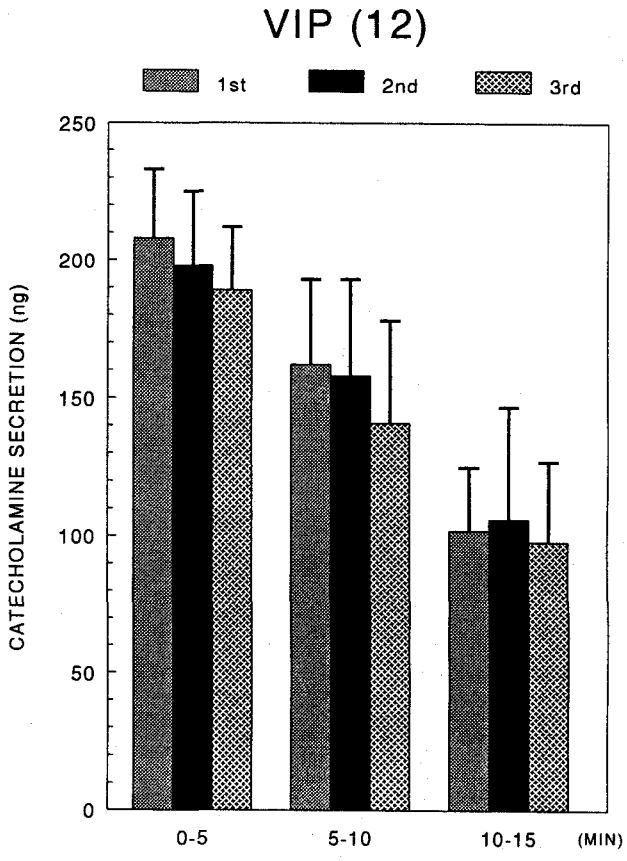

Fig. 1. The effect of repetitive administration of vasoactive intestinal polypeptide (VIP) on catecholamine secretion in the isolate perfused rat adrenal gland. VIP (3×10^{-6} M) was perfused into an adrenal vein for 5 min at the two-hour interval about 60 min after the beginning of perfused with Krebs solution. These results were obtained from 20 rat adrenal glands. Abscissa: concentration of VIP in M. Ordinate: secretion of catecholamines in ng for 5 min. The vertical columns and bars donote means and the standard errors of the corresponding means, respectively. VIP: vasoactive intestinal polypeptide. Number in the upper bracket indicates the number of animals used in the experiments. There was no significant difference between the repetitive groups.

In order to examine the tachyphylaxis to releasing effects of CA evoked by VIP, VIP at the concentration of 3×10^{-6} M was given into the perfusion stream for 5 min three times consecutively at 120 min intervals, respectively. In 12 rat adrenal glands, the CA secretions of each perfusion of VIP for 0~5 min were 208±25 ng (Ist), 198±27 ng (2nd) and 189±23 ng (3rd), those for 5~10 min were 162±31 ng (1st), 158±35 ng (2nd) and 141±37 ng (3rd), and those for 10~15 min were 102±23 ng (1st), 106±41 ng (2nd) and 98±29 ng (3rd), respectively. There was no statistically significant difference between each period of VIP administration as shown in Fig. 1.

Effect of atropine on VIP-evoked CA secretion

In order to observe the effect of muscarinic receptors, it would be interesting to examine the effect of atropine on CA release evoked by VIP and ACh. In the present work, the CA output induced by VIP or ACh was not affected in the rat adrenal gland preloaded with 3×10^{-6}M-atropine for 20 min. In 7 rat adrenal glands, 3×10^{-6} M VIP-evoked CA releases after pretreatment with atropine were 177±34 ng (0~5 min), 139±45 ng (5~10 min) and 78±12 ng (10~15 min) as compared with their control secretions of 206±23 ng (0~5 min), 106±42 ng (10~15 min) and 87±39 ng (10~15 min), respectively as shown in Fig. 2. There was no statistical significance in difference between the control responses and atropine-treated responses in VIP-induced CA secretion.

Effect of chlorisondamine on VIP-evoked CA secretion

In order to certify the effect of chlorisondamine, a selective nicotinic receptor antagonist (Taylor, 1995) on VIP-induced CA release, the rat adrenal gland was preloaded with 10^{-6}M chlorisondamine for 20 min before VIP or ACh was introduced. In the presence of chlorisondamine effect, the CA outputs evoked by perfusion with VIP (3×10^{-6} M) for 5 min

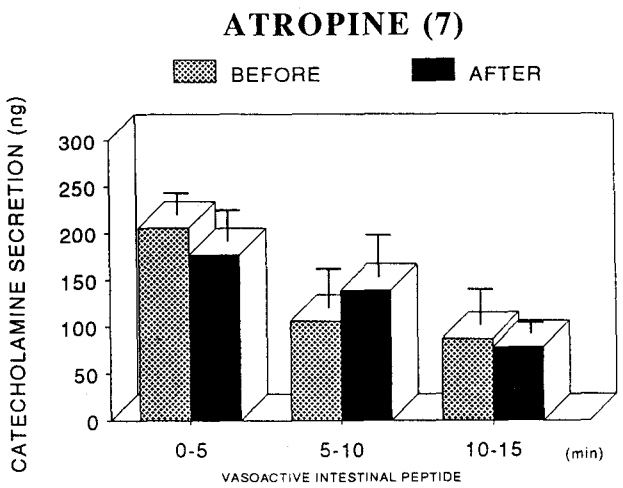

Fig. 2. The effect of atropine on the secretion of CA evoked by VIP in the perfused rat adrenal gland. Secretion of catecholamines was evoked 20 min after perfusion of adrenal gland with Krebs solution containing 3×10^{-8} M-atropine. "BEFORE" and "AFTER" indicate amounts of catecholamine released by VIP-Krebs before and after the preloading with atropine. Statistical differences were compared between amounts of catecholamines evoked by VIP (3×10^{-6} M) AFTER and BEFORE pretreatment. The statistical difference was obtained by comparing the secretory effect of CA evoked by VIP after preloading with atropine with its corresponding control. Other legends are the same as in Fig. 1.

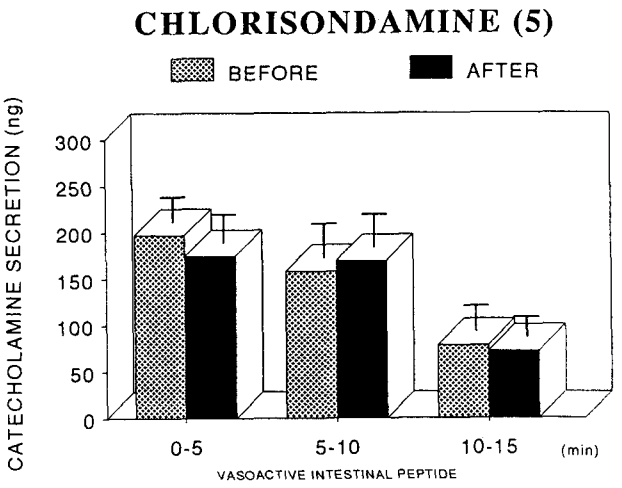

Fig. 3. The effect of chlorisondamine on the secretion of CA evoked by VIP in the perfused rat adrenal gland. Secretion of CA was produced 20 min after perfusion with Krebs solution sontaining 10^{-6} M chlorisondamine. Other legends are the same as in Fig. 1 and 2.

amounted to 174±31 ng (0~5 min), 169±36 ng (5~10 min) and 72±22 ng (10~15 min) as compared with their corresponding control releases of 197 ±27 ng (0~5 min), 158±37 ng (10~15 min) and 78±28 ng (10~15 min) from 5 experiments, respectively. There was no significant difference between groups before and after chlorisondamine-treatment. Fig. 3 illustrates the effect of chlorisondamine on CA release evoked by VIP.

The effect of naloxone on VIP-evoked CA secretion

Since it is known that opiate antagonists facilitate the splanchnic nerve stimulation-induced CA secretion from the adrenal gland of anesthetized dogs (Costa et al, 1983; Kimura et al, 1988) and reverse the inhibition of the DMPP-stimulated CA secretion evoked by etorphine, an opiate agonist from the bovine adrenal gland (Barron & Hexum, 1986) while it has been reported that naloxone, an opiate antagonist, inhibits markedly nicotinic agonist-induced CA release from the perfused rat adrenal gland (Lim et al, 1992), it is exciting to test the influence of naloxone on VIP-evoked CA outputs from the perfused rat adrenal medulla. After naloxone (1.22×10^{-7} M) was preloaded into the adrenal gland for 30 min, CA releases evoked by VIP (3×10^{-6} M) were markedly attenuated to 31±5 ng (0~5 min, $P < 0.01$), 98±22 ng (5~10 min, $P < 0.05$) and 51±12 ng (10~15 min, $P < 0.01$) as compared to their corresponding control releases of 207±62 ng (0~5 min), 170±5 ng (5~10 min) and 82±18 ng (10~15 min) from 5 rat glands, respectively (Fig. 4).

The effect of VIP-antagonist on VIP-evoked CA secretion

It was tried to investigate the influence of VIP-antagonist on VIP-induced CA release from the perfused rat adrenal glands, since it has been noted that secretion of CA evoked at low frequency (0.5~1 Hz) was less susceptible to blocking actions of cholinergic receptor antagonists than that at higher frequencies (10 Hz) (Malhtra & Wakade, 1987a; Wakade, 1988). Under the presence of VIP-antagonist ($\langle$ Lys^1, Pro^{2,5}, Arg^{3,4}, Tyr^6 $\rangle$ -vasoactive intestinal peptide, 3×10^{-7} M) which was preloaded for 30 min, VIP (3×10^{-6} M)-evoked CA secretions were significantly attenuated to 35±4 ng (0~5 min, $P < 0.05$), 31±6 ng (5~10 min, $P < 0.01$) and 19±6 ng (10~15 min, $P < 0.01$) from 8 glands as compared

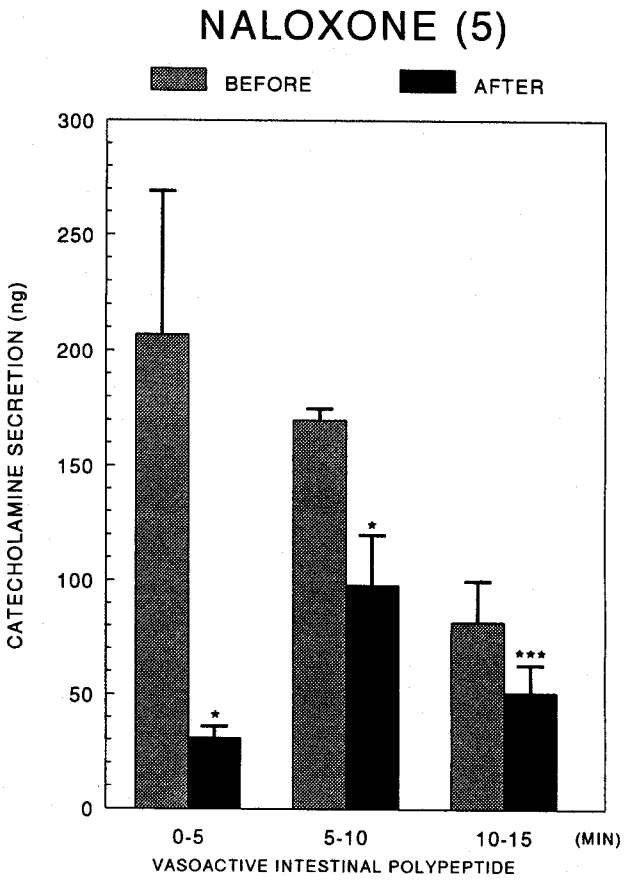

Fig. 4. The effect of naloxone on VIP-induced CA secretory responses. Naloxone (1.2×10^{-7} M) was infused for 30 min prior to administartion of VIP. Other legends are the same as in Fig. 1 and 2. *: $P < 0.05$, ***: $P < 0.01$.

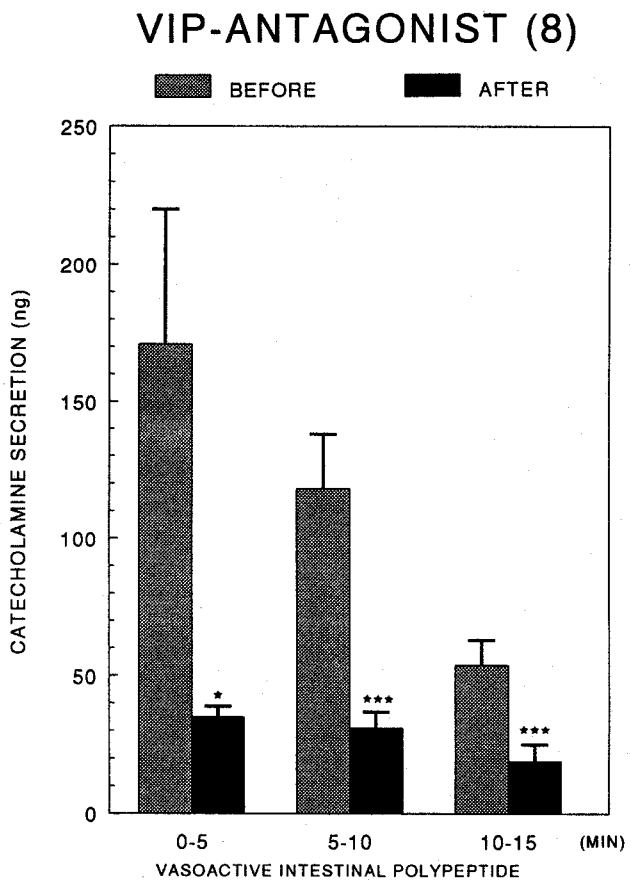

Fig. 5. The effect of VIP-antagonist on CA secretion evoked by VIP. VIP-antagonist ($\langle$ Lys^1, Pro^{2,5}, Arg^{3,4}, Tyr^6 $\rangle$ -vasoactive intestinal peptide, 3×10^{-7} M) was perfused into the adrenal gland 30 min before introducing VIP. Other legends are the same as in Fig. 1 and 2. *: $P < 0.05$, ***: $P < 0.01$.

with their corresponding control releases of 171 ± 49 ng (0~5 min), 118 ± 20 ng (5~10 min) and 54 ± 9 ng (10~15 min), respectively. Fig. 5 illustrates the inhibitory responses of VIP-antagonist on VIP-induced CA secretion.

The effect of perfusion with calcium-free Krebs on VIP-evoked CA secretion

Since it is found that the physiological release of CA and dopamine-beta -hydroxylase from the perfused cat adrenal gland is dependent on the extracellular calcium concentration (Dixon, Garcia & Kirpekar, 1975), it is of particular interest to test whether the secretory effect induced by VIP is also related to extracellular calcium ions. Thus, the adrenal gland was preperfused with calcium-free Krebs solution for 30 min prior to introduction of VIP. In absence of extracellular calcium, CA releases by VIP (3×10^{-6} M) were significantly blocked to 94 ± 27 ng (0~5 min, $P < 0.05$), 29 ± 7 ng (5~10 min, $P < 0.05$) and 12 ± 3 ng (10~15 min, $P < 0.05$) from 6 rat glands as compared with their corresponding control responses of 186 ± 24 ng 0~5 min), 103 ± 21 ng (5~10 min) and 70 ± 23 ng (10~15 min) as shown in Fig. 6.

The effect of nicardipine on VIP-evoked CA secretion

In order to investigate the effect of nicardipine, a dihydropyridine derivative and L-type Ca^{++} channel blocker (Gilman et al, 1991) on VIP-evoked CA secretion, nicardipine (10^{-6} M) was preloaded into the adrenal gland for 30 min. In the presence of nicardipine effect, CA releases induced by perfusion of VIP (3×10^{-6} M) for 5 min were greatly depressed

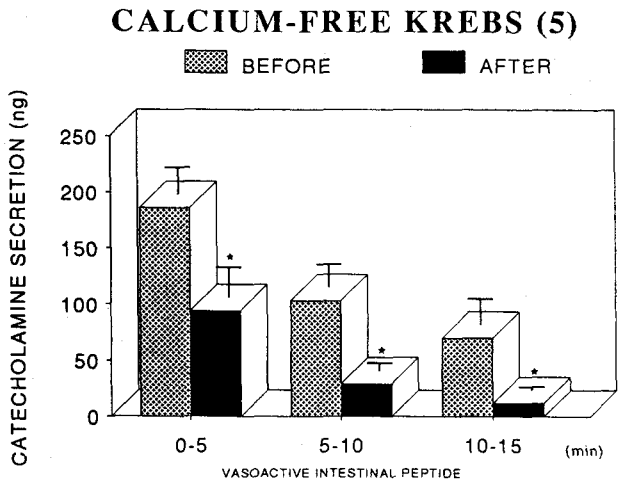

Fig. 6. The effect of Ca^{2+}-free Krebs solution on the secretion of CA evoked by VIP in the perfused rat adrenal gland. Secretion of CA was induced 30 min following perfusion of adrenal gland with Ca^{2+}-free Krebs solution. Other legends are the same as in Fig. 1 and 2. *: P ⟨ 0.05.

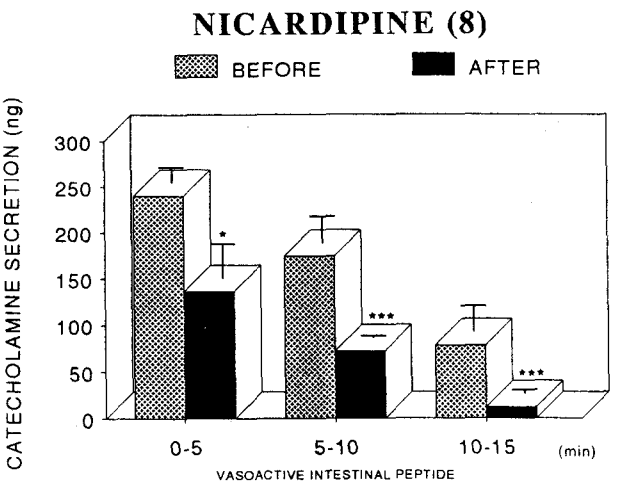

Fig. 7. The effect of nicardipine on VIP-evoked CA secretory responses. Nicardipine (10^{-6} M) was perfused for 30 min before introducing VIP. Other legends are the same as in Fig. 1 and 2. *: P ⟨ 0.05, ***: P ⟨ 0.01.

to 136±37 ng (0~5 min, P ⟨ 0.05), 72±22 ng (5~10 min, P ⟨ 0.01) and 12±4 ng (10~15 min, P ⟨ 0.01) from 8 rat glands as compared with their corresponding control responses of 240±17 ng (0~5 min), 174±29 ng (5~10 min) and 78±28 ng (10~15 min), respectively. Fig. 7 illustrates that nicardipine inhibits CA secretory responses evoked by VIP.

The effect of TMB-8 on VIP-evoked CA release

Since it has been reported that muscarinic, but not nicotinic activation causes CA secretion independent of extracellular calcium in the perfused adrenal glands of the cat (Nakazato et al, 1988), suggesting that the presence of an intracellular calcium pool linked to a muscarinic receptors, and that TMB-8, an intracellular calcium antagonist, inhibits both nicotinic and muscarinic stimulation-induced CA release in the rat adrenal glands (Lim et al, 1992), an attempt was made to test the TMB-8 on VIP-evoked CA secretion. In 8 rat adrenal glands, CA secretions evoked by 5 min-perfusion of VIP (3×10^{-6} M) after preloading with TMB-8 (10^{-5} M) for 30min were clearly blocked to 87±16 ng (0~5 min, P ⟨ 0.05), 78±28 ng (5~10 min, P ⟨ 0.01) and 30±6 ng (10~15 min, P ⟨ 0.05) from 8 rat glands in comparison with their corresponding control responses of 176±37 ng (0~5 min), 122±29 ng (5~10 min) and 56±

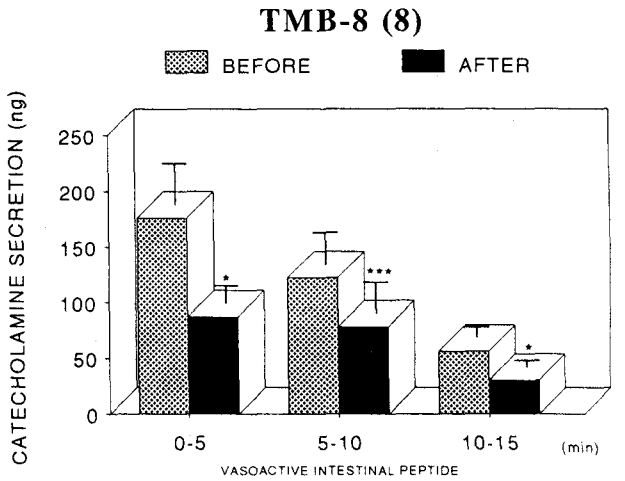

Fig. 8. The effect of TMB-8 on CA secretion evoked by VIP. TMB-8 (10^{-5} M) was given into the perfusion stream for 30 min after obtaining the corresponding control responses of VIP. Other legends are the same as in Fig. 1 and 3. *: P ⟨ 0.05, ***: P ⟨ 0.01.

10 ng (10~15 min), respectively as shown in Fig. 8.

The effect of VIP infusion on ACh- and high K^+ -evoked releases

In terms of the facts that VIP-induced CA release was markedly inhibited by the pretreatment of atropine and chlorisondamine as in Fig. 2 and 3, it is of interest to examine the influence of VIP per-

fusion on ACh- and high K^+-induced CA secretions. The adrenal gland was preloaded with VIP (3×10^{-6} M) for 20 min before ACh- and high K^+ were introduced. In the presence of VIP effect, ACh-induced CA output was greatly enhanced to 884 ± 85 ng ($P < 0.01$, n=10) for 4 min as compared to its corresponding CA secretion of 679 ± 45 ng/4 min prior to perfusion with VIP shown in table 10. In the present work, in the absence of VIP, high K^+ (5.6×10^{-4} M)-evoked CA secretion was 971 ± 114 ng (0~4 min) while in the presence of VIP (3×10^{-6} M) which was perfused into the gland for 20 min high K^+-evoked CA secretion was prominently potentiated to 1248 ± 155 ng (0~4 min, $P < 0.01$) from 7 adrenal glands. Fig. 9 illustrates the augmenting effect of VIP perfusion on the secretory responses induced by ACh and high K^+.

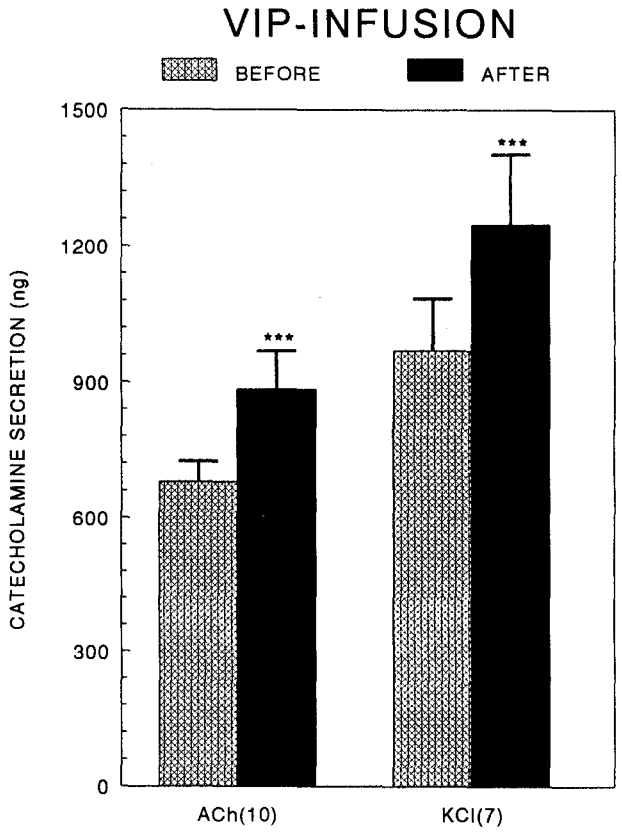

Fig. 9. The effect of VIP-infusion on CA secretion evoked by ACh and excess K^+. VIP (3×10^{-6} M) was perfused for 20 min after obtaining the control response of excess K^+- and ACh-evoked CA secretory responses. "BEFORE" and "AFTER" indicate amounts of catecholamine released by excess K^+- and ACh before and after the preloading with VIP-Krebs. Other legends are the same as in Fig. 1 and 2. ***: $P < 0.01$.

DISCUSSION

The results of the present investigation suggest strongly that VIP causes an increased CA secretion in a calcium-dependent fashion through the stimulation of VIPergic receptors from the isolated perfused rat adrenal glands. It also seems that theses findings may provide the crucial piece of evidence for VIP as a neurotransmitter in the adrenal medullary synapse and VIP may be the non-cholinergic excitatory substance. In the present study, the facts that VIP causes a significant increase in CA secretion from the rat adrenal glands are in agreement with the previous works performed on similar preparations (Malhotra & Wakade, 1987a; Wakade, 1988; Wakade et al, 1991; Yamaguchi, 1993). The major criteria that VIP may cause CA secretion by the activation of VIPergic receptor, and that it is a noncholinergic neurotransmitter in adrenal medulla are as follows.

Firstly, stimulatory effect of VIP was antagonized by VIP antagonist. In the present investigation, it was successful in demonstrating the antagonistic action of the VIP antagonist on CA secretion evoked by the administered VIP. Secretion by VIP was reduced by more than averagely 27% of the control release. This finding indicates strongly that VIP-induced CA releasing effect is mediated via stimulation of receptor in the adrenomedullary chromaffin cells. In support of this idea, Wakade and his colleagues (1991) have found that VIP receptor antagonist inhibits VIP-evoked CA secretion, without affecting ACh-evoked secretion. It has also been found that VIP receptors are present on cultured bovine chromaffin cells and cause a cyclic AMP response in these cells (Wilson, 1988).

Secondly, in the present experiments, the stimulatory effect of exogenous VIP in CA secretion was not abolished by the pretreatment of atropine and chlorisondamine, whereas ACh-induced CA release greatly depressed by them. Although it is beyond the scope of this study to discuss precise mechanisms, together with the previous reports (Wakade et al, 1991; Malhotra & Wakade, 1986; 1987a), the present observations are consistent with the view that VIP-induced adrenal CA secretion is mediated at least by a nonnicotinic mechanism, because atropine and chlorisondamine are known to be a specific muscarinic and nicotinic antagonist, respectively, at the level of nicotinic and muscarinic receptors in autonomic ganglia as well as adrenal medullary

chromaffin cells (Taylor, 1995). This pharmacological evidence is consistent with the idea that the rat adrenal medulla has a VIPergic component that are distinct from the cholinergic receptors. In support such a proposal, VIP-like immunoreactive nerve fibers have been demonstrated in the adrenal medullary synapse (Hokfelt et al, 1981; Holzwarth, 1984; Maubert et al, 1990; Yoshikawa et al, 1990) and VIP has been also localized not only in splanchnic nerve terminals but also in adrenal chromaffin cells (Carmicheal & Stoddard, 1993; Kondo, 1985; Linnoila et al, 1980; Said, 1984). Moreover, the present findings demonstrate that the potentiating effect of VIP on high K^+- and ACh-evoked CA release, appear to be mediated through the activation of other unknown receptors, suggesting strongly that there exists a specific VIPergic receptors on the rat adrenomeddulary chromaffin cells.

Thirdly, one of the unexpected findings in the present investigation was that the stimulatory effects of VIP on CA secretion were blocked markedly by pretreatment with naloxone, an opioid-receptor antagonist. How does naloxone block the effect of VIP? One possiblity is that VIP releases another agent from the adrenal gland which activates naloxone -sensitive receptors of the chromaffin cells to evoke the secretion of CA. Another possibility is that the high concentrations of naloxone can directly affect the function of VIPergic receptors in a non-specific manner to suppress the secretion. High concentrations of naloxone are known to interfere with the function of non-opioid receptors (Dingledine et al, 1978; Dean et al, 1982; Malhotra & Wakade, 1987). There should be a species difference between the rat and bovine adrenal glands in the pharmacology of the non-cholinergic component. Malhotra & Wakade (1987a) have shown that non-cholinergic component is inhibited by high concentration (30 μM) of naloxone, which does not inhibit the response to ACh at this concentration. However, at the concentration (12 μM) used in the present study, both VIP- and ACh-evoked CA secretory responses were significantly depressed. It is unlikely that the non-cholinergic component in rat is mediated by opioid peptides released from the splanchnic nerve terminals, since opioid peptides had only a very weak stimulatory effect on CA secretion from the rat adrenal glands (Malhotra & Wakade, 1987b) and no specific opioid receptors were detected in rat adrenals by radioligand binding sites (Bunn et al, 1988a). Specific high-affinity opioid receptors are present in bovine adrenal medulla and on bovine chromaffin cells (Kumakura et al, 1988; Bunn et al, 1988a), and high levels of opioid peptides are present in the bovine adrenal gland, including nerve terminals innervating chromaffin cells (Schultzberg et al, 1978; Lewis & Stern, 1983). It has been also reported that opioid antagonist, including naltrexone and naloxone, inhibit the nicotine-induced CA secretion from the adrenal chromaffin cells in culture (Lemaire et al, 1981; Dean et al, 1982; Marley et al, 1986a), and that naloxone also depresses ACh- and DMPP-evoked CA secretion in the perfused rat adrenal glands (Lim et al, 1992). In terms of these findings, the present experimental results that naloxone markedly inhibits VIP-evoked CA release in the perfused rat adrenal gland suggest strongly that the inhibitory effect of nicotinic stimulation-evoked CA secretion by naloxone may be exerted through the other unknown mechanism in addition to the opiate antagonism. Opioid receptors have been shown to exist in the chromaffin cells and to play a significant role in modulating CA release (Saiani & Guidotti, 1982). Most opioid receptors in adrenal medullary cells are κ -receptors (Castanas et al, 1983; 1985a; 1985b).

Fourthly, generally, the indispensible role of calcium in the neurosecretory process has been well established. Yet, according to the assumptions of Baker & Knight (1978; 1980), the relationship between the concentration of intracellular calcium and the transmitter release has not been determined in nerve terminals. As mentioned above, calcium plays the crucial role in process of depolarization-neurotransmitter release coupling in many types of secretory cells (Douglas, 1968; Schultz & Stolze, 1980; Williams, 1980). In the present work, removal of extracellular Ca^{++} inhibited greatly CA secretion evoked by VIP of ACh. The secretory effect of CA by VIP is apparently dependent on extracellular calcium. However, in the present experiment, the reason for considerable response to VIP is not clear. It may be that chromaffin cells of the rat adrenal gland contain an intracellular store of calcium which participates in the secretion of CA as shown in the bovine adrenal gland (Baker & Knight, 1978). Such a store may not be easily depleted by mere removal of extracellular calcium. Some investigators (Boxler, 1968; Ohashi et al, 1974; Casteels & Raeymeakers, 1979; Malagodi & Chiou, 1974; Takahara et al, 1990) reported that intracellular stores of calcium have been

shown to play some roles in contraction of smooth muscle produced by noradrenaline of ACh in Ca^{2+} -free medium. Moreover, in the present study, the finding that considerable response to VIP is still remained in the presence of a Ca^{++}-channel blocker nicardipine although the secretory effect of CA evoked by VIP is significantly diminished by pre-treatment with Ca^{2+}-entry blocker may support above results. In addition, in terms of the fact that pre-administration of TMB-8 inhibited clearly CA secretion evoked by VIP as well as that by ACh in the present perfused rat adrenal gland, it is felt that VIP-evoked CA release may be exerted at least partly through mobilization of calcium from the intracellular store located within chromaffin cells. TMB-8 [3. 4. 5-trimethoxybenzoate-8-(N. N-diethylamino) octylester], a benzoic acid derivative is known well to act by preventing mobilization of calcium from intracellular stores without altering Ca^{2+} influx into stores (Charo et al, 1976; Chiou & Malagodi, 1975; Rubin et al, 1980; Smith & Idea, 1979; Wiedenkeller & Sharp, 1984). In support of the present results, Yamada and his colleagues (1988) have found that the secretory effect of CA evoked by caffeine is inhibited by TMB-8 from perfused cat adrenal glands in the absence of extracellular calcium. The similar results were also obtained from the rat adrenal gland (Lim et al, 1991; Lim et al, 1992). Moreover, it is known that ACh and pilocarpine cause a partial increase CA release from both guinea pig adrenal glands (Nakazato et al, 1984) and perfused cat adrenal glands (Nakazato et al, 1988) with Ca^{2+}-free lock solution. At least a part of the VIP-evoked CA secretion is induced by a sustained intracellular calcium rise due to the continuous Ca^{2+} influx through Ca^{2+} channels. These channels are sensitive to dihydropyridine, but do not appear to be identical to the L-type voltage-sensitive Ca^{2+} channel. Taken together, the present experimental results demonstrate that CA secretion evoked by VIP is exerted through the stimulation of VIPergic receptors in the rat adrenal gland, and that this VIP's secretory effect is mediated at least partly by the liberation of Ca^{++} from the internal stores.

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