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PITUITARY ADENYLATE CYCLASE ACTIVATING POLYPEPTIDE PROVOKES CULTURED RAT CHROMAFFIN CELLS TO SECRETE ADRENALINE

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Summary: Pituitary adenylate cyclase activating polypeptide (PACAP) provoked the rat chromaffin cells to secrete adrenaline. Within 20 min, the amount of adrenaline secreted by PACAP (10^{-8}M) was as much as that caused by acetylcholine (10^{-4}M). PACAP, but not acetylcholine, induced a long-term (over 120 min) increase in secretion of adrenaline. PACAP also activated adenylate cyclase and elevated cytosolic Ca^{2+} concentration. Furthermore, we found immunoreactive PACAP and PACAP binding sites in the rat adrenal medulla. These results suggest that PACAP has an important role in stimulating secretion of adrenaline in the adrenal medulla.

The main functions of chromaffin cells of the adrenal medulla are synthesis, storage and secretion of catecholamines (1-3). Concerning secretion, acetylcholine is thought to be an important inducer. A second messenger induced by acetylcholine is Ca^{2+}, and the role of Ca^{2+} on the exocytosis of catecholamine is well established (3,4). It was demonstrated that tyrosine hydroxylase, a rate-limiting enzyme of the biosynthesis of catecholamines (5), is activated by phosphorylation with cAMP-dependent protein kinase (6). Moreover, tyrosine hydroxylase gene has a cAMP responsive element in the promoter region (7) and elevation of cAMP induces tyrosine hydroxylase in PC12 cells (8).

PACAP was isolated from the ovine hypothalamus based on its ability to stimulate adenylate cyclase in rat anterior pituitary cells (9). It was reported that two PACAP forms exist. One is a C-terminal amidated and 38 residues peptide named PACAP38, the other is a C-terminal amidated and the N-terminal 27 amino acids of PACAP38, named PACAP27 (10). These two forms were revealed to be equally potent in stimulating adenylate cyclase (10). Molecular

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The abbreviations used are: PACAP38, pituitary adenylate cyclase activating polypeptide with 38 residues; PACAP27, a shorter form peptide with 27 residues corresponding to the N-terminal 27 amino acids of PACAP38; VIP, vasoactive intestinal polypeptide; $[Ca^{2+}]_i$, cytosolic free Ca2+ concentration ; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; EGTA, [Ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HBSS-Hepes Hanks' balanced salt solution containing 0.05% bovine serum albumin and 10mM Hepes , pH 7.4 .

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cloning revealed that their structures are conserved in ovines, humans (11) and rats (12). Interestingly, PACAP is 1000 times more potent than VIP in stimulating adenylate cyclase (9), although PACAP is highly homologous to vasoactive intestinal polypeptide (VIP) (68% homology with N-terminus 28 residues). PACAP binding sites were observed in the anterior pituitary, lung, liver, duodenum, ovary, and thymus of the rat (13), central nervous system of the bovine (14) and rat (15). Moreover, PACAP activated adenylate cyclases in membranes of the hippocampus, pancreas (16), and liver (17) of the rat, and in rat astrocyte cells (18), human neuroblastoma cells NB-OK (19) and rat cancerous pancreatic acinar cells AR 4-2J (20).

We previously demonstrated that PACAP activated adenylate cyclase in PC12h via specific PACAP binding sites, which showed a high affinity for PACAP but not for VIP (21). Since PC12h cells are derived from chromaffin cells of the adrenal medulla, we further investigated role for PACAP in the adrenal medulla in the present study. Using a primary culture of the rat adrenal medulla, we examined effects of PACAP on adenylate cyclase activities, secretion of adrenaline and cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$). We also investigated the amounts of endogenous PACAP and its binding sites in the rat adrenal medulla by means of enzyme immunoassay and quantitative autoradiography, respectively.

Materials and Methods

Materials-Newborn calf serum was purchased from Mitsubishi Chemical Industries (Tokyo, Japan), 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes), [Ethylenebis (oxyethylenenitrilo)]- tetraacetic acid (EGTA), EDTA and fura-2 from Dojin (Kumamoto, Japan), cAMP assay kit from Amersham (Buckinghamshire, England), and human VIP from the Peptide Institute (Osaka, Japan). PACAP38 and PACAP27 were synthesized by an automatic synthesizer (ABI model 430A). PACAP27 and VIP were iodinated by the peroxydase method as described previously (14).

Cell isolation and culture-Chromaffin cells were isolated from male Sprague-Dawley rats (13 weeks old) by enzymatic digestion (22) in Hanks' balanced salt solution containing 0.05% bovine serum albumin and 10 mM Hepes, pH 7.4 (HBSS-Hepes). Isolated cells were plated on collagen-coated 24-multiwell plates or cover glasses with Dulbecco modified Eagle medium/ Ham's F12 (1/1) containing 5% newborn calf serum, 5% horse serum, 1 μM cytosine arabinoside and antibiotics. The cells were incubated in a humidified atmosphere of 95% air and 5% CO_2 for 3 to 5 days.

Measurement of cAMP and catecholamine-Replacing the medium in the 24-multiwell plate with 500 μl of HBSS-Hepes buffer, the cells were preincubated for 30 min at 37°C and then PACAP, acetylcholine or VIP was added. After the incubation at 37°C, aliquots of the medium were examined. In order to measure the long-term effects, the cells were washed twice with HBSS-Hepes and incubated further in the absence of PACAP or acetylcholine without a reagent. For cAMP assay, the aliquots were applied directly to radioimmunoassay (cAMP assay kit, Amersham). For catecholamine assay, the aliquotes were acidified by acetic acid and applied to HPLC equipped with an electrochemical detector. Catecholamine separation was done isocratically as follows: column, ODS-120T (TOSOH,Tokyo, Japan); mobile phase, 40 mM citrate-CH_3COOH (pH 3.5), 230 mg/l 1-octanesulfonic acid sodium salt, 5 mg/l EDTA(2Na), 17% methanol; flow rate, 0.8 ml/min. The condition of electrochemical detector, EC-8011 (TOSOH), was as follows: working electrode, a glassy carbon; reference electrode, Ag/AgCl; a working electrode potential, 750 mV; temperature, 25°C.

Measurement of $[Ca^{2+}]_i$-The cells on collagen coated cover glasses were used for a single cell $[Ca^{2+}]_i$ measurement. Replacing the medium with HBSS-Hepes including 4 μm fura-2/AM, the cells were incubated at 25°C for 1 h. Washing the cells twice with HBSS-Hepes, $[Ca^{2+}]_i$ was measured in the same buffer with the microspectroscopic system (M-500, INTER DEC, Osaka, Japan). Excitation was dual-wavelength (340 nm and 380 nm) illumination with a grating scanner (scanning rate, 20 Hz) for monochromator and emission wavelength was 510 nm.

Autoradiography of PACAP binding sites-In vitro autoradiography was carried out according to the method previously described (15). Male Sprague-Dawley rats (250 g) were decapitated and their adrenal glands were immediately removed and frozen with dry ice. The tissues were cut into 20 μm-thick sections on cryostat at -15°C and mounted onto gelatin-coated slide glasses. The sections were incubated at 22°C in 50 mM Tris-HCl buffer, pH 7.4 containing 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1% bovine serum albumin, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 20 μg/ml leupeptin and 0.05% 3-[(3-cholamido-propyl)dimetylammonio]-1-propanesulfonate with 0.1 nM [^{125}I]PACAP or 0.1 nM [^{125}I]VIP. Adjacent sections were incubated in the presence of 10^{-6}M unlabeled PACAP27 or VIP for the examination of nonspecific binding. Following incubation, they were washed twice for 15 min in 50 mM Tris-HCl buffer, pH 7.4 at 4°C and subjected to autoradiography. Quantitations of binding sites were assessed with an image analyzer (BAS200 bioimaging analyzer, Fuji photo film, Japan) using autoradiographic [^{125}I]micro-scale (Amersham, England).

Enzyme immunoassay for endogenous PACAP-Adrenal glands obtained above were cut into 300-μm-thick serial sections on cryostat at -10°C, mounted onto slide glasses. Medullae and cortices were separately punched out from adrenal slices in a cold box at -20°C. Tissues were homogenized in 200 μl of 1 M acetic acid containing 20 mM HCl and 10 μg/ml of pepstatin. Homogenates were immediately boiled for 10 min and centrifuged at 25,000 X g for 30 min at 4°C. Aliquotes of the supernatant were processed to enzyme immunoassay for PACAP38 according to the method of Matsumoto *et al.* (23).

Results

As shown in Fig. 1A, PACAP38 caused an increase in secretion of cAMP from the chromaffin cells. Extracellular cAMP level was dose-dependently raised and reached a maximum level (1.3 pmol/10^5cells) at 10^{-8}M of PACAP38. The potency of PACAP27 was the same as that of PACAP38, while VIP was around 1000 times less potent than those of PACAPs in stimulating cAMP production. Furthermore, PACAPs enhanced secretion of adrenaline from the chromaffin cells (Fig. 1B). PACAP38 and PACAP27 had the same potency, while VIP was very weak.

Extracellular cAMP level was increased by PACAP38 (10^{-8}M), but not by acetylcholine (10^{-4} M) within the first 20 min (Fig. 2A). Furthermore, PACAP, but not acetylcholine, induced a long-term (over 120 min) increase of extracellular cAMP level (Fig. 2A). The amount of

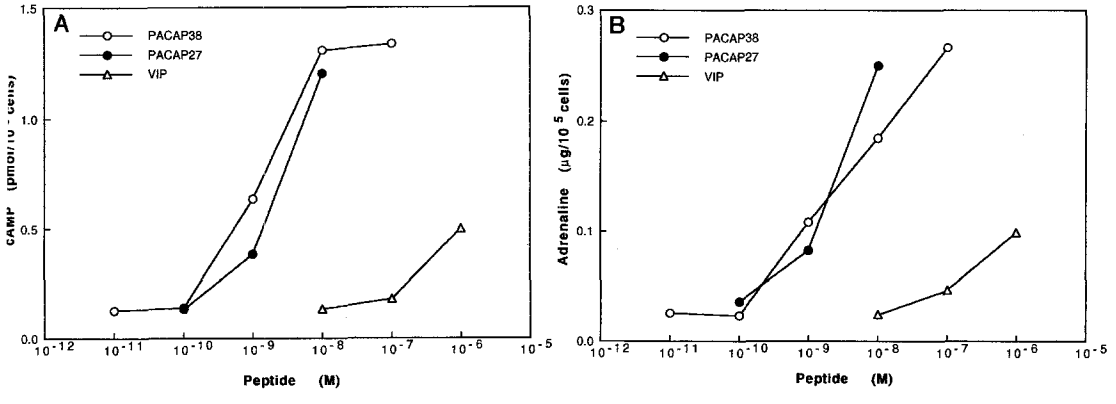

Fig. 1. Dose-dependent secretion of cAMP (A) and adrenaline (B) by PACAP27, PACAP38 and VIP in the cultured chromaffin cells for 2 hr. The same aliquots were used to measure cAMP and adrenaline. Each point is the average of three experiments.

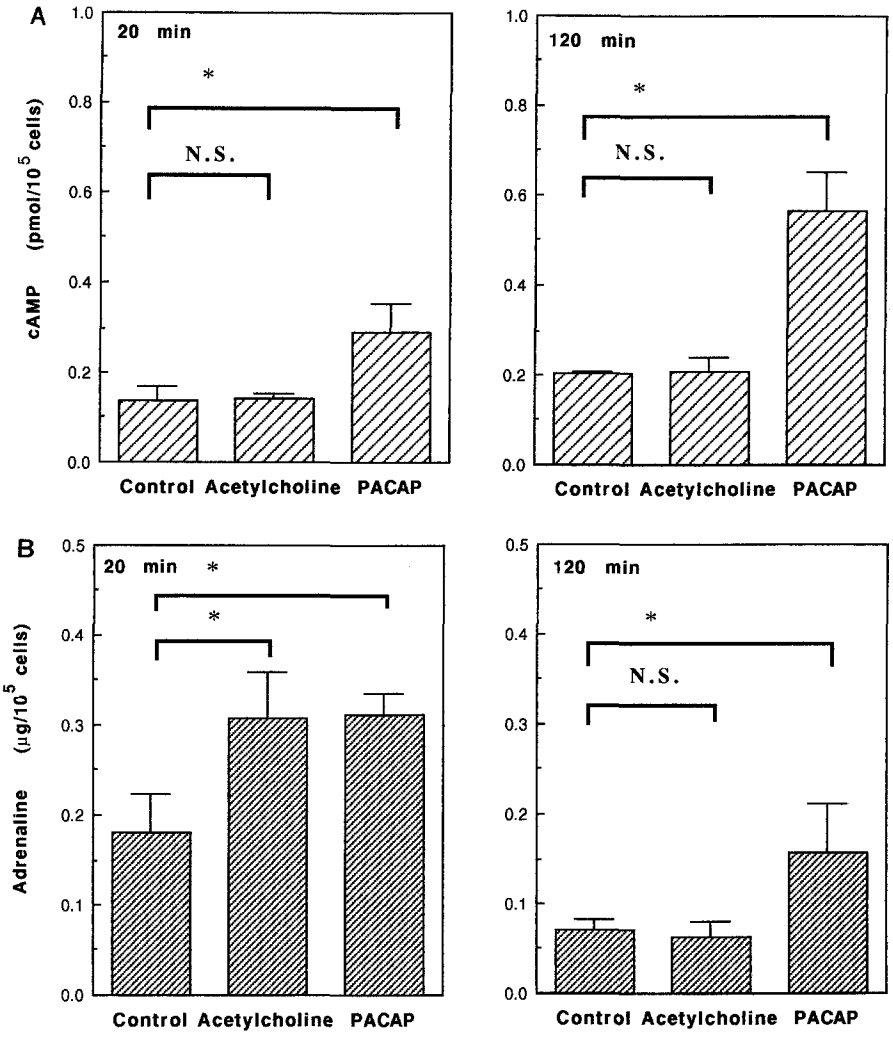

Fig. 2. Effect of PACAP38(10^{-8}M) and acetylcholine(10^{-4}M) on cAMP (A) and adrenaline (B) secretion in the chromaffin cell cultures. Each point represents the mean±SEM of three independent experiments. *: significantly different at $P<0.05$ between the indicated columns (Student's t-test).

adrenaline secreted by 10^{-8}M of PACAP38 was similar to that caused by 10^{-4}M of acetylcholine for initial 20-min incubation (Fig. 2B). A significant increase in adrenaline release was still observed following a 120-min incubation after PACAP was removed (Fig. 2B).

We measured $[Ca^{2+}]_i$ of a single cell by means of a microspectroscopic system. Fig. 3 shows the time course of change in the ratio of fluorescence intensity at 340 nm to that at 380 nm in the fura-2 loaded single chromaffin cell. PACAP38 (10^{-7}M) raised $[Ca^{2+}]_i$ of the chromaffin cell in two-phase manner (transient and sustained phases) (Fig. 3A). The sustained phase caused by PACAP38 was abolished by EGTA (Fig. 3B). In contrast, the transient phase was little affected by the addition of EGTA (Fig. 3B). Acetylcholine induced an increase in $[Ca^{2+}]_i$ only transiently (Fig. 3C).

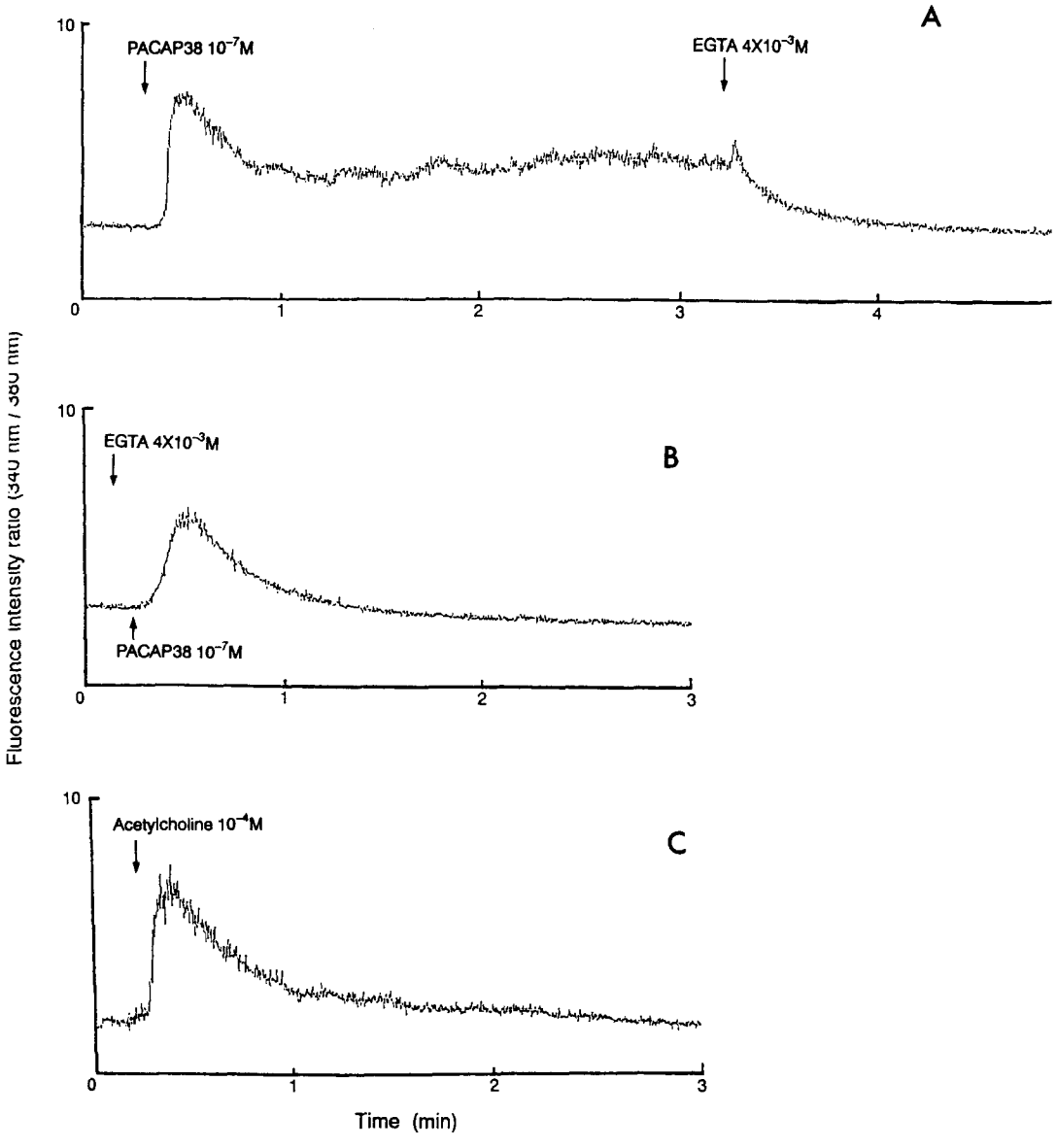

Fig. 3. Time-course changes in $[Ca^{2+}]_i$ of a single chromaffin cell induced by PACAP38 (A,B) or acetylcholine (C). The ratio of fluorescence intensity was measured with the microspectroscopic system.

Fig. 4 shows autoradiograms of PACAP binding sites in the adrenal gland. High densities of PACAP binding sites were observed in the medulla. As shown in Fig. 5, densities of specific PACAP binding sites in the medulla were 12 times higher than those in the cortex, while significant difference was not observed in VIP binding site densities between in these two regions. In the medulla, PACAP binding site densities were 5 times higher than those of VIP. Recently, Shivers, *et al.* also showed the existence of PACAP binding sites in the adrenal medulla (24).

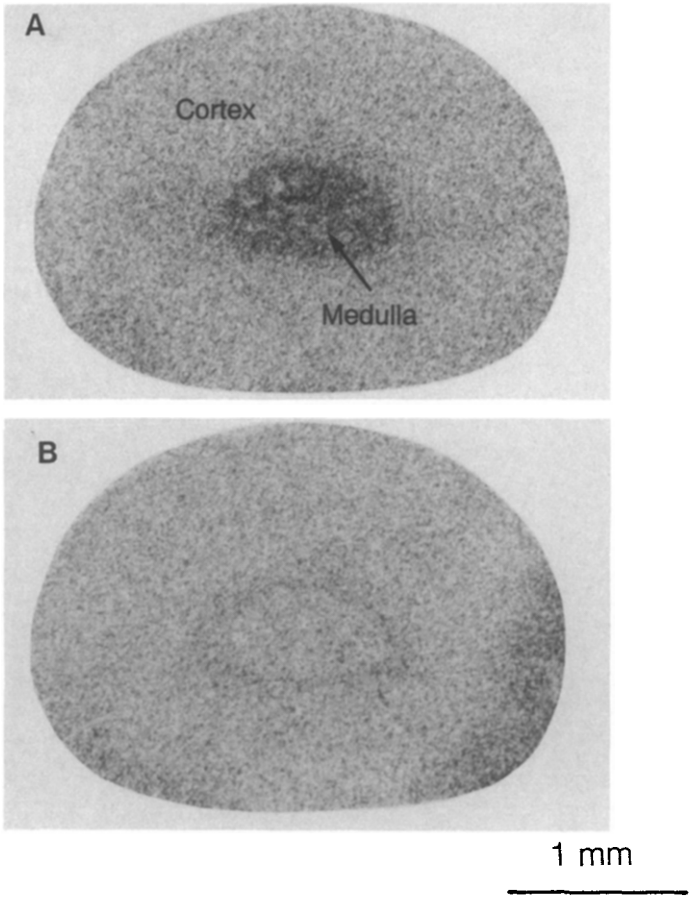

Fig. 4. Autoradiograms of [^{125}I]PACAP27 binding sites in the rat adrenal gland. Tissue sections were incubated with 10^{-10} M [^{125}I]PACAP27 (A) or incubated in the presence of 10^{-6} M unlabeled PACAP27 (B). Film autoradiograms were obtained by the apposition of radiolabeled sections to Hyperfilm (Amersham, England) for 12 days.

Endogenous PACAP level in the adrenal medulla was 24-fold higher than that in the cortex (Table 1). The cross reactivity for VIP of the antibody used for this enzyme immunoassay was less than 0.1% (data not shown).

Discussion

The present study demonstrated for the first time that PACAP provoked the rat chromaffin cells to secrete adrenaline. Within 20 min, the amount of adrenaline release induced by PACAP was comparable with that of acetylcholine. Furthermore, PACAP, but not acetylcholine, caused a long-term (over 120 min) increase in the secretion of adrenaline. We also found that PACAP activated adenylate cyclase and provoked $[Ca^{2+}]_i$ increase in the rat chromaffin cells at 10^{-9} M. These activities of PACAP for the rat chromaffin cells were 1000 times more potent than those of VIP, although PACAP is structurally similar to VIP.

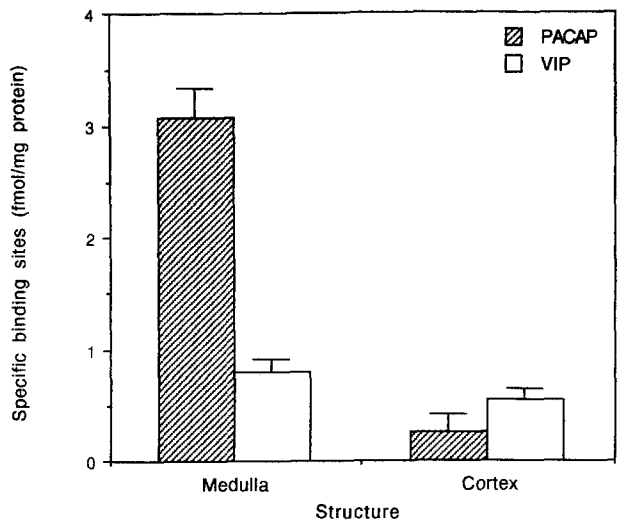

Fig. 5. Densities of specific binding sites for [^{125}I]PACAP27 in the adrenal gland, measured with autoradiography using an image analyzer. Each value represents the mean ± SEM of 14 independent experiments.

Acetylcholine released from terminals of its innerving cholinergic sympathetic nerve fibers has been thought to be the main secretogogue of catecholamine from the chromaffin cells. The second messenger induced by acetylcholine is Ca^{2+} and the role of Ca^{2+} is well established (4). We found that PACAP also increased $[Ca^{2+}]_i$ in the chromaffin cells. The elevation of $[Ca^{2+}]_i$ induced by PACAP occurred in a dual phase manner, while acetylcholine raised $[Ca^{2+}]_i$ only transiently under our experimental conditions. The transient phase was caused through the release of internal Ca^{2+} store because it increased irrespective of EGTA. This phase was considered to evoke the exocytotic secretion. In this respect, PACAP might have the same function as acetylcholine in releasing catecholamines from the intracellular store. The sustained phase of $[Ca^{2+}]_i$ increase caused by PACAP was brought about by the mobilization of extracellular Ca^{2+}, since EGTA in the extracellular solution abolished this phase. However, it is still ambiguous whether PACAP activates Ca^{2+} channel directly or indirectly to cause Ca^{2+} influx.

PACAP, but not acetylcholine, provoked a long-lasting (over 120 min) increase in secretion of adrenaline. One possible reason for this finding is an activation of catecholamine synthesis by

TABLE 1. Endogenous PACAP levels in adrenal gland

Structure	Tissue weight(mg)	PACAP (pg/mg tissue)	number of determination
Medulla	2.4	*201.1+21.3	6
Cortex	12.4	*8.3+ 0.5	6

* Mean ± SEM

PACAP . PACAP could activate adenylate cyclase in the chromaffin cells. It was demonstrated that tyrosine hydroxylase was activated by phosphorylation with cAMP-dependent protein kinase (6). The stimulation of cAMP synthesis by PACAP would result in an activation of tyrosine hydroxylase via phosphorylation. Further, the sustained increase of $[Ca^{2+}]_i$ induced by PACAP may enhance the exocytosis of catecholamines from the chromaffin cells over 120 min. It remains to be studied whether PACAP activates two second messenger pathways, namely cAMP and Ca^{2+}, independently or activates only a single second messenger pathway and another pathway is concomitant.

Malhotra and Wakade (25) demonstrated that, besides acetylcholine, non-cholinergic substance(s) of splanchnic nerves also mediated neurally evoked secretion of catecholamines in the isolated perfused adrenal gland of the rat. VIP has been suggested to be this non-cholinergic substance (26-28). Interestingly, we observed that PACAP was 1000 times more potent than VIP in catecholamine secretion. Furthermore, we revealed the existence of endogenous PACAP and its binding sites in the rat adrenal medulla. These findings suggest that PACAP is an important non-cholinergic secretogogue in the adrenal medulla.

In the adrenal medulla, physiological stress provokes an increase in tyrosine hydroxylase activity following increase in cAMP (29, 30) and interruption of the splanchnic nerves supplying the adrenal glands prevents this tyrosine hydroxylase induction (31). Moreover, tyrosine hydroxylase gene had a cAMP responsive element in the promoter region (7) and VIP was shown to induce tyrosine hydroxylase protein in PC12 cells for over 2 days (8). Although some of cholinergic nerve fibers contain VIP-like immunoreactivity in that structure (32, 33), we found that VIP was very weak in stimulating cAMP production in adrenal medulla cells as compared to PACAP. These results suggest that PACAP rather than VIP has an important role in a long-lasting (over 2 days) induction of tyrosine hydroxylase by stimulating cAMP second messenger pathway.

The present study clearly suggests that PACAP has an important physiological role in adrenaline secretion from the rat adrenal medulla as a non-cholinergic secretogogue.

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