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ADRENAL PHEOCHROMOCYTOMA PC12H CELLS RESPOND TO PITUITARY ADENYLATE CYCLASE ACTIVATING POLYPEPTIDE

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SUMMARY: An adrenal pheochromocytoma cell line, PC12h, was found to respond to a novel hypothalamic neuropeptide, Pituitary Adenylate Cyclase Activating Polypeptide (PACAP). The cells elevated both intracellular and extracellular cAMP levels on stimulation by PACAP, whereas they showed little response to VIP which is structurally related to PACAP. Using [^{125}I]PACAP27 (a shorter form of the peptide) and [^{125}I]VIP, we found large amounts of specific binding sites for PACAP but few binding sites for VIP in PC12h cells. These results indicate that PC12h cells respond to PACAP via a specific PACAP receptor.

The PC12 cell line is derived from a transplantable rat adrenal pheochromocytoma (1). Tischler *et al.* (2) and Roskoski *et al.* (3) have reported that VIP stimulates the adenylate cyclase activity of PC12 cells. However, the activation of the adenylate cyclase required an unphysiologically high concentration of VIP (1 μM or more). This observation suggests that a real physiological modulator for the PC12 cells exists and it might be a VIP related peptide rather than VIP itself.

Recently, Miyata *et al.* isolated two novel peptides closely related to VIP from ovine hypothalamic tissues, named Pitui-

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Abbreviations: PACAP38, pituitary adenylate cyclase activating polypeptide with 38 residues and amidated C-terminus; PACAP27, a shorter form peptide with 27 residues corresponding to the N-terminal 27 amino acids of PACAP38 and amidated C-terminus; VIP, vasoactive intestinal polypeptide; HBSS, Hanks' balanced salt solution; HEPES, N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid; CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate; BSA, bovine serum albumin.

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tary Adenylate Cyclase Activating Polypeptide, with 38 residues (PACAP38) (4), and the other one with the N-terminus 27 residues of PACAP38 (PACAP27) (5). Their structures are highly homologous to that of VIP (68% homology with N-terminus 28 residues) and are revealed to be conserved in humans and rats (6).

The present study was undertaken to test a hypothesis that PACAP is a physiological modulator for the adenylate cyclase activity of PC12 cells. We demonstrate that a nanomolar concentration of PACAP38 or PACAP27 stimulated the adenylate cyclase of PC12h cells (a subclone of PC12 cells (7)), whereas a micromolar concentration of VIP was required to obtain the same extent of the response. Moreover, we found large amounts of PACAP specific binding sites, but, in contrast, few VIP binding sites in PC12h cells. Based on this evidence, we discuss the physiological function of PACAP in a peripheral tissue, such as the adrenal glands.

MATERIALS AND METHODS

Materials: PC12h cells (7) were kindly provided by Dr. Hatanaka (Institute for Protein Research, Osaka University). Newborn calf serum was purchased from Mitsubishi Chemical Industries (Tokyo, Japan), cAMP assay kits from Amersham (Buckinghamshire, England), [^{125}I]VIP (2200 Ci/mmol) from New England Nuclear (Boston, MA), and human VIP from the Peptide Institute (Osaka, Japan).
Peptide synthesis and radioiodination: PACAP38 and PACAP27 was synthesized by an automatic synthesizer (ABI model 430A). PACAP27 was iodinated by the peroxidase method as described previously (8).
Cyclic AMP assay: PC12h cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. For the cAMP assay, cells were plated on collagen-coated 48-well multiwell plates at about 5 x 10^4 cells/well. The cells were used at 7 to 10 days after plating. The medium was replaced with 500 µl of HBSS including 0.05% BSA. Following incubation at 37°C for 30 min, PACAP or VIP was challenged to the cells. After the incubation at 37°C, the cells were washed twice with HBSS and then intracellular cAMP was extracted with 20% perchloric acid. The extract was neutralized with 1.5M KOH containing 60mM HEPES and subjected to radioimmunoassay. Extracellular cAMP was assayed by applying aliquots of the incubated medium directly.
Binding assay: Cells in 24-well multiwell plates were washed with HBSS including 0.05% BSA and 0.05% CHAPS (binding buffer) and incubated at 37°C for 1 hr in the presence of 30 pM [^{125}I]PACAP or [^{125}I]VIP in 200 µl of the binding buffer. After the

completion of the binding, the cells were washed with the same buffer and then solubilized with 500 µl of 0.5 M NaOH and aliquots were counted by a gamma-counter.

RESULTS

Exposure of PC12h cells to PACAP38 resulted in a rapid elevation of an intracellular cAMP (10 times that of the control at 8 min), reaching its maximal level (20 times that of the control) by 25 min and the level did not reduce to baseline within 60 min (Fig. 1). This sustained increase of intracellular cAMP was dose-dependent and reached 100 times that of the control level at 10^{-6} M of PACAP38 (Fig. 2). The minimum effective dose of PACAP38 was 10^{-10} M. The potency of PACAP27 was the same as PACAP38 (Fig. 2). Compared to PACAP, VIP was very weak (about 1000-fold weaker) in stimulating cAMP production.

Furthermore, PC12h cells secreted cAMP by the addition of PACAP. As indicated in Fig. 3, extracellular cAMP slowly increased and reached a plateau level by 120 min. This plateau level increased dose-dependently and reached 200 times that of the control level at 10^{-7} M of PACAP38 (Fig. 4). As in the

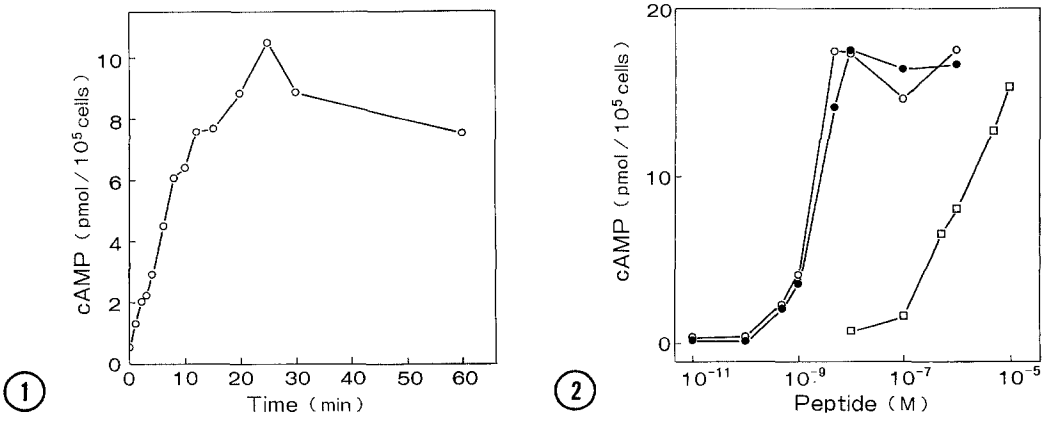
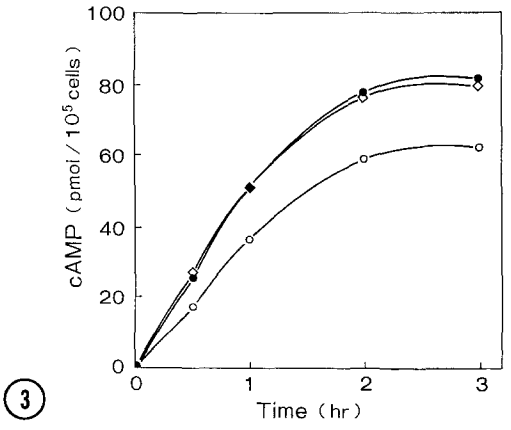


Fig. 1. Time course of intracellular cAMP accumulation in PC12h cells after addition of 10 nM PACAP38.

Fig. 2. Dose dependency of intracellular cAMP accumulation in PC12h cells during 20 min incubation with PACAP38(●), PACAP27(○) or VIP(□).



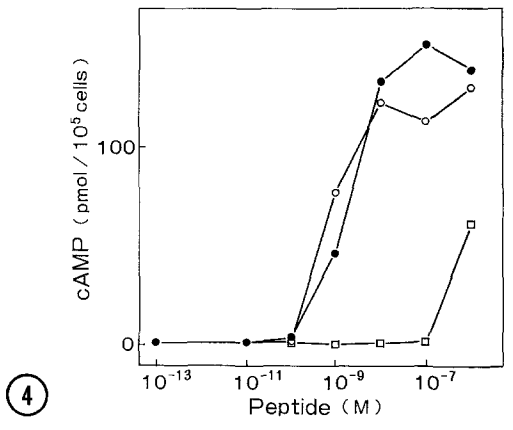
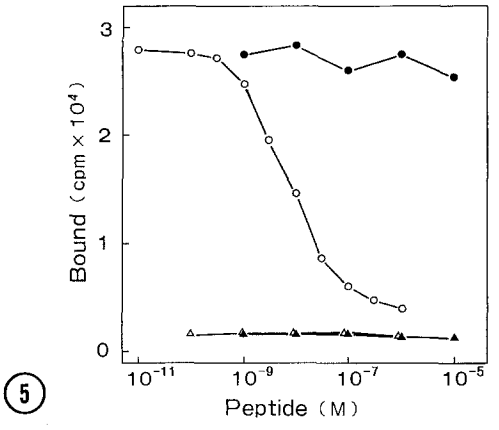


Fig. 3. Time course of cAMP secretion from PC12h cells with 10^{-7}M (●), 10^{-8}M(◇) or 10^{-9}M(○) PACAP27.

Fig. 4. Dose dependency of cAMP secretion from PC12h cells during 2 hr incubation with PACAP38(●), PACAP27(○) or VIP(□).

intracellular cAMP production, both PACAP38 and PACAP27 showed similar activities to extracellular cAMP accumulation while VIP was rather ineffective.

We attempted to characterize the receptor of PACAP in PC12h cells by receptor binding experiments. We used [^{125}I]PACAP27 for binding experiments instead of [^{125}I]-PACAP38 because the latter was adhesive to cells and the substratum. The previous investigation confirmed that PACAP27 shows similar affinity to a brain PACAP receptor as PACAP38 (8). Competitive binding curves of [^{125}I]PACAP27 and of [^{125}I]VIP are shown in Fig. 5. PACAP27 displaced the binding of [^{125}I]PACAP27 potently, however, VIP failed to inhibit the binding of [^{125}I]PACAP27 even at the high concentration of 10^{-5}M. Scatchard plot representation of the competition curve of [^{125}I]PACAP27 with PACAP27 is shown in Fig. 6. The plot showed the presence of at least two different binding sites. For the high affinity binding site, K_d=2.1 nM and B_{max}=3.2 X 10^5 sites/cell were obtained. The low affinity binding site was considered a nonphysiological binding site and not studied further. Although the same amount of radioactivity was added, an extremely small amount of specific [^{125}I]VIP binding was observed.



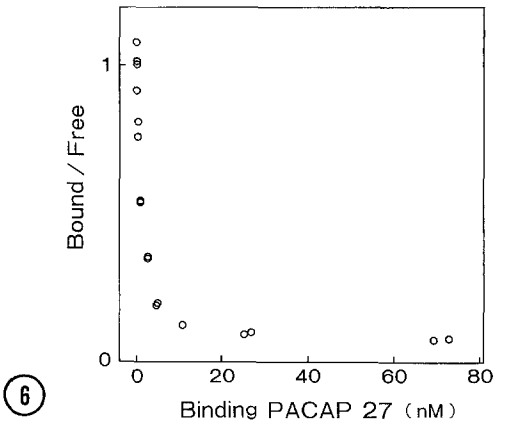


Fig. 5. Inhibition of [^{125}I]PACAP27 or [^{125}I]VIP binding to PC12h cells by increasing concentration of unlabeled PACAP27 or VIP: [^{125}I]PACAP vs. PACAP27 (○), [^{125}I]PACAP27 vs. VIP (●), [^{125}I]VIP vs. PACAP27 (△), [^{125}I]VIP vs. VIP (▲).

Fig. 6. Scatchard representation of the PACAP27 binding data shown in Fig.5.

DISCUSSION

We have demonstrated that PC12h cells respond sensitively to both PACAP38 and PACAP27, but very weakly to VIP. We also found that the PC12h cells have an abundance of PACAP specific binding sites. The binding site shares similar properties with PACAP receptor found in the central nervous system (8,9) in that it shows comparable affinity for PACAP27 and PACAP38 but scarcely interacts with VIP. Moreover, the cells did not have a significant amount of VIP binding sites. These results concerning the binding sites are compatible with the data on cyclase stimulating activities. Therefore, the high sensitivity to PACAP but not to VIP could be attributed to the predominance of specific PACAP receptors over VIP specific receptors. Weak activity of VIP might be exerted by weak interaction of VIP with the PACAP receptors.

Chromaffine cells in adrenal medulla is known to release catecholamine on stimulation by its innerving cholinergic sympathetic nerve fibers. Some of these nerve fibers contain VIP-like immunoreactivity in rats (10) and humans (11). Furthermore, VIP

stimulates the secretion of catecholamines from the rat adrenal medulla (12). In vitro, PC12 cells originating from adrenal medulla respond to a higher concentration of VIP and increase the production of catecholamines (2,3). From this study using a subclone of PC12 cell line, it is suggested that the response of PC12 cells to VIP is mediated via PACAP receptors rather than VIP receptors. Moreover, Gottschall *et al.* reported that the specific binding of [^{125}I]PACAP, but no [^{125}I]VIP binding, was observed in adrenal membrane fractions (9). Considering this, we offer the possibility that PACAP participates in the regulation of the functions of adrenal medulla.

Buscail *et al.* reported that PACAP stimulates adenylate cyclase in the rat pancreatic acinar cell line, AR 4-2J, with similar potency to that in PC12h cells (13). Furthermore, the ligand specificity of PACAP receptors in these cells is similar to that of PC12h PACAP receptors. The present report, in addition to their observation, indicates that functional PACAP receptors are distributed, not only in the central nervous system, but also in some peripheral tissues, such as the pancreas and adrenal gland. The distribution of PACAP immunoreactivity in these tissues is now under investigation.

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