Pituitary Adenylate Cyclase-Activating Polypeptide Stimulates the Synthesis of Dopamine in Cultured Bovine Adrenal Chromaffin Cells

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The effect of pituitary adenylate cyclase-activating polypeptide₁₋₃₈ (PACAP₁₋₃₈) on the synthesis of dopamine in cultured bovine adrenal chromaffin cells was examined. PACAP₁₋₃₈ stimulated [¹⁴C]dopamine synthesis from [¹⁴C]tyrosine, in a concentration-dependent manner, causing maximal stimulation at 10⁻⁷M. This stimulatory action of PACAP₁₋₃₈ was not significantly inhibited by staurosporine (an inhibitor of protein kinase C) or in the cells in which protein kinase C was down-regulated by prolonged exposure to TPA (an activator of protein kinase C), whereas it was partially attenuated in Ca²⁺-free medium. PACAP₁₋₃₈ increased the formation of [³H] inositol phosphates, [Ca²⁺]_i, ⁴⁵Ca²⁺ uptake and cAMP level. The peptide also stimulated the phosphorylation of tyrosine hydroxylase, the enzyme catalyzing the rate-limiting step in dopamine synthesis. Dopamine synthesis and tyrosine hydroxylase phosphorylation stimulated by the maximal effective concentration of dibutyryl cAMP or high K⁺, which activates Ca²⁺ uptake, were further enhanced by PACAP₁₋₃₈. These results indicate that PACAP₁₋₃₈ may stimulate the activities of cAMP- and calcium-dependent protein kinases in cultured bovine adrenal chromaffin cells, resulting in increase in the synthesis of dopamine probably by stimulation of phosphorylation of tyrosine hydroxylase. (*Hypertens Res* 1995; 18 Suppl. 1: S169-S171)

Key Words: PACAP, dopamine synthesis, tyrosine hydroxylase, phosphorylation, chromaffin cell

The neuropeptide pituitary adenylate cyclase-activating polypeptides (PACAP) originally isolated from ovine hypothalamus stimulates adenylate cyclase activation in rat cultured pituitary cells (1). In addition to brain, PACAP is widely distributed in other organs such as the testis, gastrointestinal tract, lung and adrenal gland (1). In bovine adrenal chromaffin cells, the phosphorylation and activation of tyrosine hydroxylase (the rate-limiting enzyme in dopamine synthesis) are mediated by cAMP- and calcium-dependent protein kinases (2). We have reported the activation and phosphorylation of tyrosine hydroxylase by physiological stimulation in adrenal chromaffin cells and pheochromocytoma PC-12 cells (3-6). However, little is known about the effect of PACAP on dopamine biosynthesis. In the present study, we investigated the effects of PACAP₁₋₃₈ on synthesis of dopamine and phosphorylation of tyrosine hydroxylase in cultured bovine adrenal chromaffin cells.

Methods

Bovine adrenal chromaffin cells were dispersed enzymatically and cultured as described previously (7). For measurement of dopamine synthesis, it was measured as described previously (4). The cells were incubated at 37°C for 15 to 45 min in 1 ml of bal-

anced salt solution (BSS) containing 20 μM L-[¹⁴C]tyrosine (0.5 μCi). [¹⁴C]Dopamine from L-[¹⁴C]tyrosine was isolated on aluminium hydroxide, and radioactivity was counted by liquid scintillation spectrometry (8). For measurement of inositol phosphates formation, the cells were incubated for 48 h with 5 μCi/ml [³H]inositol in inositol-free Dulbecco's modified Eagle's serum-free medium. Then the cells were preincubated in BSS containing 10 mM LiCl for 10 min at 37°C and incubated for 10 min with or without test compounds. [³H]inositol phosphates was measured using anion-exchange chromatography as described previously (9). For measurement of intracellular free calcium ([Ca²⁺]_i), the [Ca²⁺]_i in single chromaffin cells was measured using the fluorescent Ca²⁺ indicator fura-2. Fluorescence was measured in single chromaffin cell on the cover glass using a fluorescence spectromicroscope (excitation, 340 / 380 nm, emission, 510 nm). The [Ca²⁺]_i was calculated using the equation described previously (10). For measurement of ⁴⁵Ca²⁺ uptake, incubation medium containing 6 μCi of ⁴⁵Ca²⁺ was added to the cells. After incubation, the dishes were immediately chilled on ice and washed three times with ice-cold Ca²⁺-free BSS. The ⁴⁵Ca²⁺ taken up into the cells was extracted with 1% Triton X-100 and counted in a liquid scintillation counter. For measurement of cyclic AMP

levels, 3-isobutyl-1-methylxanthine (0.5 mM) was added to all tissue culture dishes to prevent the breakdown of cAMP. The cAMP level was measured by enzyme immunoassay with an Amersham cAMP enzyme immunoassay system kit. For measurement of phosphorylation of tyrosine hydroxylase, it was measured as described previously (6). The phosphorylated tyrosine hydroxylase was separated by antiserum specific for tyrosine hydroxylase. Then, the phosphorylated tyrosine hydroxylase was applied to SDS-polyacrylamide slab gel as described previously (11).

Results and Discussion

The synthesis of [^{14}C]dopamine from [^{14}C]tyrosine caused by various concentrations of PACAP $_{1-38}$ was examined. PACAP $_{1-38}$, at concentrations between of 10^{-9} and 10^{-7}M , stimulated [^{14}C]dopamine synthesis in a concentration-dependent manner, and its maximal effect occurred at 10^{-7}M (control, 0.22 ± 0.02 nmol/30 min/dish; 10^{-7}M PACAP $_{1-38}$ -treated cells, 0.42 ± 0.03 nmol/30 min/dish). PACAP $_{1-38}$, however, failed to stimulate [^{14}C]dopamine synthesis from [^{14}C]DOPA (control, 3.7 ± 0.4 nmol/30 min/dish; 10^{-7}M PACAP $_{1-38}$ -treated cells, 3.9 ± 0.4 nmol/30 min/dish), suggesting that PACAP $_{1-38}$ increased the conversion of tyrosine to DOPA catalyzed by tyrosine hydroxylase, the rate-limiting step in dopamine biosynthesis. Moreover, the stimulation of [^{14}C]dopamine synthesis by PACAP $_{1-38}$ (10^{-7}M) proceeded linearly with the duration of incubation for at least 45 min.

To know the stimulatory mode of PACAP $_{1-38}$ in [^{14}C]dopamine synthesis, we examined whether the effect of PACAP $_{1-38}$ would be attenuated in calcium-free medium. The stimulatory action of PACAP $_{1-38}$ (10^{-7}M) was significantly inhibited by calcium-free medium (43% inhibition). We then examined the possible involvement of protein kinase C in PACAP $_{1-38}$ -stimulated fashion. The stimulatory action of PACAP $_{1-38}$ (10^{-7}M) was not significantly influenced by staurosporine (10^{-6}M), an inhibitor of protein kinase C, and by deprivation of protein kinase C from the cells by prolonged (24 h) exposure to relatively high concentrations (10^{-6}M) of TPA (12). It seems that the stimulation of dopamine synthesis following PACAP $_{1-38}$ treatment is dependent, in part, on extracellular calcium, but independent of protein kinase C. High K^+ and dibutyryl cAMP also stimulated dopamine synthesis, the effect of the former (but not the latter) being absolutely dependent on extracellular calcium.

Inositol trisphosphate (IP_3) produced by the stimulation of phospholipase C is reported to mobilize Ca^{2+} from the intracellular storage pools (13). The effects of PACAP $_{1-38}$ on the level of [^3H]inositol phosphates derived from [^3H]inositol and [Ca^{2+}]i using the fluorescent Ca^{2+} indicator fura-2 were examined. PACAP $_{1-38}$ (10^{-7}M) increased [^3H]inositol phosphates by 141% over the control. PACAP $_{1-38}$ (10^{-7}M) increased [Ca^{2+}]i in a biphasic manner, and [Ca^{2+}]i attained to 238 and 618 nM at the initial rapid and a subsequent sustained phase, respec-

tively. Since PACAP $_{1-38}$ induced a biphasic rise in [Ca^{2+}]i, we examined the effect of PACAP $_{1-38}$ on $^{45}\text{Ca}^{2+}$ uptake into adrenal chromaffin cells. PACAP (10^{-7}M) and high K^+ (56 mM) increased $^{45}\text{Ca}^{2+}$ uptake by 102% and 149% over the control, respectively. These data, therefore, suggest that PACAP $_{1-38}$ stimulates the release of Ca^{2+} from intracellular pools and the uptake of extracellular Ca^{2+} on adrenal chromaffin cells. The effects of PACAP $_{1-38}$ and forskolin (an activator of adenylate cyclase) on cAMP level were carried out. PACAP $_{1-38}$ (10^{-7}M) increased cAMP to 709% of the control which was comparable to that due to forskolin.

Dibutyryl cAMP and high K^+ are reported to increase dopamine synthesis by the activation of tyrosine hydroxylase through cAMP- and calcium-dependent protein kinases, respectively (2, 14). Dibutyryl cAMP (7 mM) or high K^+ (56 mM), by itself, increased dopamine synthesis by 120 or 150%, and their simultaneous application caused additive increase (276%) in dopamine synthesis. Although PACAP $_{1-38}$ (10^{-7}M), by itself, increased dopamine synthesis by 90% and also augmented the stimulatory effects of dibutyryl cAMP and high K^+ from 120 and 152% to 196 and 228% respectively, the stimulatory effects of PACAP $_{1-38}$ in combination of dibutyryl cAMP or high K^+ were less than additive. Since the concentrations of PACAP $_{1-38}$, dibutyryl cAMP and high K^+ used were those causing the maximal stimulation of dopamine synthesis (3, 4, 6), these results suggest that PACAP $_{1-38}$ -induced dopamine synthesis may be mediated by both cAMP- and calcium-dependent protein kinases. Furthermore, phosphorylation of tyrosine hydroxylase was stimulated 2.5 fold in the cells treated with PACAP $_{1-38}$ (10^{-7}M). In addition, PACAP $_{1-38}$ further enhanced the stimulatory effects of dibutyryl cAMP (7 mM) and high K^+ (56 mM) from 2.2 and 3.6 fold to 3.3 and 4.1 fold respectively.

Thus, these results suggest that PACAP $_{1-38}$ may activate cAMP- and calcium-dependent protein kinases in cultured bovine adrenal chromaffin cells, resulting in increase in the dopamine synthesis mediated by stimulation of tyrosine hydroxylase phosphorylation and its activation.

References

1. Harmar T, Lutz E: Multiple receptor for PACAP and VIP. *TIPS* 1994; **15**: 97-99.
2. Masserano JM, Vulliet PR, Tank AW, Weiner N: The role of tyrosine hydroxylase in the regulation of catecholamine synthesis, in Trendelenburg U, Weiner N (eds): *Handbook of Experimental Pharmacology; Catecholamines*, Berlin, Springer, 1990, pp. 427-469.
3. Houchi H, Nakagawa H, Oka M, Watanabe J, Isosaki M, Ohuchi T: Increase in cyclic AMP level and stimulation of catecholamine synthesis in isolated bovine adrenal medullary cells caused by forskolin. *Jpn J Pharmacol* 1984; **36**: 97-99.
4. Houchi H, Nakanishi A, Uddin MM, Ohuchi T, Oka M: Phorbol ester stimulates catecholamine synthesis in isolated bovine adrenal medullary cells. *FEBS Lett* 1985; **188**: 205-208.
5. Houchi H, Oka M, Misbahuddin M, Morita K, Nakanishi A: Stimulation by vasoactive intestinal

- polypeptide of catecholamine synthesis in isolated bovine adrenal chromaffin cells. *Biochem Pharmacol* 1987; **36**: 1551-1554.
6. Houchi H, Masserano JM, Bowyer JF, Weiner N: Regulation of tyrosine hydroxylase activity in pheochromocytoma PC-12 cells by bradykinin. *Mol Pharmacol* 1990; **37**: 104-110.
 7. Oka M, Isosaki M, Yanagihara N: Isolated bovine adrenal medullary cells: studies on regulation of catecholamine synthesis and release, in Usdin E, Kopin IJ, Barchas J (eds): Catecholamines; Basic and Clinical Frontiers. Oxford, Pergamon Press, 1979, pp. 70-72.
 8. Matsuoka M: Function and metabolism of catecholamines in the brain. *Jpn J Pharmacol* 1964; **14**: 181-193.
 9. Berridge MJ, Downes CP, Hanley MR: Lithium amplifies agonist-dependent phosphatidylinositol response in brain and salivary glands. *Biochem J* 1982; **206**: 587-595.
 10. Gryniewicz G, Poenie M, Tsien RY: A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 1985; **260**: 3440-3450.
 11. Yanagihara N, Tank AW, Weiner N: Relationship between activation and phosphorylation of tyrosine hydroxylase by 56 mM K^{+} in PC-12 cells in culture. *Mol Pharmacol* 1984; **26**: 141-147.
 12. Grove DS, Mastro AM: Prevention of the TPA-mediated down-regulation of protein kinase C. *Biochem Biophys Res Commun* 1988; **151**: 94-99.
 13. Berridge MJ, Irvine RF: Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature (Lond)* 1984; **312**: 315-321.
 14. Tachikawa E, Tank AW, Yanagihara N, Mosimann W, Weiner N: Phosphorylation of tyrosine hydroxylase on at least three sites in rat pheochromocytoma PC-12 cells treated with 56 mM K^{+} : determination of the sites on tyrosine hydroxylase phosphorylated by cyclic AMP-dependent and calcium/calmodulin-dependent protein kinases. *Mol Pharmacol* 1986; **30**: 476-485.