Role of calcium channels and adenylate cyclase in the PACAP-induced adrenal catecholamine secretion

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Received 13 November 2000; accepted in final form 26 March 2001

The aim of this study is to clarify the contribution of VDCCs and adenylate cyclase to the PACAP-induced adrenal catecholamine secretion. We examined the effects of calcium-free solution, an L-type VDCC blocker nifedipine, an N-type VDCC blocker ω-conotoxin GVIA, a P/Q-type VDCC blocker ω-conotoxin MVIIC, and an adenylate cyclase inhibitor MDL-12330A on the PACAP-induced catecholamine secretion in the intact adrenal gland. The PACAP-induced adrenal catecholamine secretion is suggested to require the influx of extracellular calcium (13, 23). PACAP may also activate adenylate cyclase to enhance adrenal catecholamine secretion (23). However, contribution of these mechanisms to the PACAP-induced adrenal catecholamine secretion remains unclear. Whereas L-, N-, and Q-type voltage-dependent calcium channels (VDCCs) participate in the PACAP-induced catecholamine secretion (20), PACAP increases calcium influx via nicardipine-sensitive (L-type), but not N-, P-, or Q-type, VDCCs (27), in bovine cultured adrenal chromaffin cells. In rat cultured adrenal chromaffin cells, neither L- nor N-type VDCCs participate in the PACAP-induced catecholamine secretion (23), and participation of other types of VDCCs is unknown. PACAP elevates intracellular cAMP in bovine (22) and rat (23) cultured adrenal chromaffin cells, and inhibition of protein kinase A suppresses the PACAP-induced catecholamine secretion in rat cultured adrenal chromaffin cells (23). However, an adenylate cyclase activator, forskolin, which elevates intracellular cAMP, does not induce catecholamine secretion in bovine chromaffin cells (31). In addition to these inconsistencies observed in cultured adrenal chromaffin cells, little is known about the role of and relation between adenylate cyclase and each type of VDCC in the PACAP-induced catecholamine secretion in the intact adrenal gland.

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sociation between drug effects on adrenal epinephrine (Epi) and norepinephrine (NE) secretion has been reported (16, 18), the PACAP-induced Epi and NE secretion might involve different pathways. In this regard, we determined changes in Epi and NE output.

MATERIALS AND METHODS

Animal preparation. All procedures for handling animals were approved by the Animal Experimentation Committee of Tohoku University Graduate School of Pharmaceutical Sciences. Male Wistar rats, weighing 220–360 g, were housed at 21–24°C and maintained on a standard diet and water ad libitum. Rats were anesthetized with pentobarbital sodium (50 mg/kg ip). The surgical procedure was described previously (19). A polyethylene cannula, used for perfusion of the adrenal gland, was inserted into the adrenal vein through the renal vein. Then the adrenal gland was carefully removed from the animal, and a small slit was made into the adrenal cortex just opposite the entrance of the adrenal vein. Perfusion of the adrenal gland was started, to ensure that no leak was present, and the perfusate escaped only from the slit of the adrenal gland. The adrenal gland was placed in a water-jacketed chamber, the temperature of which was maintained at 37°C with a thermostatically controlled water circulator (model NTT-1200, EYELA, Tokyo, Japan). After extraction of the adrenal gland, the animal was killed by exsanguination.

Perfusion of the adrenal gland. The adrenal gland was perfused by means of a peristaltic pump (model MP-3A, EYELA) at a rate of 0.02 ml/min. The perfusion was carried out with Krebs-Henseleit solution of the following composition (in mM): 118 NaCl, 4.7 KCl, 1.2 MgSO4, 2.6 CaCl2, 1.2 KH2PO4, 24.9 NaHCO3, and 11.1 glucose. Krebs-Henseleit solution was maintained at 37°C by the thermostated bath and bubbled with 95% O2-5% CO2. Perfusion stream through a branching polyethylene catheter solution was maintained at 37°C by the thermostated bath and bubbled with 95% O2-5% CO2. Perfusion samples were collected in chilled tubes containing 50 μl of 0.1 M perchloric acid to prevent oxidation of catecholamines.

PACAP infusion. PACAP solution was infused into the perfusion stream through a branching polyethylene catheter by using a microsyringe pump (model CMA/200, Bioanalytical Systems, West Lafayette, IN) at a rate of 0.02 ml/min. The PACAP infusion was performed for 3 min in each experimental period. The calculated concentration of PACAP in the perfusate was 100 nM.

Experimental protocols. In preliminary experiments, we observed that catecholamine (Epi and NE) secretion from the adrenal gland in response to infusion of PACAP (100 nM) increased as the infusion was repeatedly applied. Therefore, in this study, infusion of 100 nM PACAP was twice performed for 5 min at 10-min intervals before the start of experiments to obtain stable catecholamine output responses. We confirmed that, after these conditioning infusion periods, catecholamine secretion responses induced by 100 nM PACAP for 3 min were stable in four consecutive experimental periods (10-min intervals). The PACAP-induced increases in Epi output were 116.9 ± 11.9, 113.3 ± 11.8, 108.2 ± 9.2, and 104.0 ± 9.5 ng/min (n = 10), and the PACAP-induced increases in NE output were 18.3 ± 2.6, 18.4 ± 2.5, 17.5 ± 2.3, and 17.8 ± 2.5 ng/min (n = 10) in the first, second, third, and fourth experimental periods, respectively. In group 1 (n = 9), the effects of perfusion with calcium-free solution on the PACAP-induced increases in catecholamine output were examined. The first infusion of PACAP (after the conditioning infusion periods) was regarded as control (1st trial). Perfusion with calcium-free solution was started 5 min before the start of the second trial. In group 2 (n = 7), the effects of nifedipine (1 and 3 μM) on increases in catecholamine output induced by PACAP were examined. The first trial was regarded as control. Perfusion with nifedipine-containing Krebs-Henseleit solution was started 5 min before the start of the second and third trials, respectively. In groups 3 (n = 10), 4 (n = 8), and 5 (n = 8), the effects of ω-conotoxin GVIA (10 and 100 nM), ω-conotoxin MVIIIC (100 and 1,000 nM), and MDL-12330A (30 and 100 μM), respectively, on increases in catecholamine output induced by PACAP were examined with the same protocol used for group 1. In groups 6 (n = 10), 7 (n = 6), and 8 (n = 10), the effects of nifedipine (3 μM), MDL-12330A (100 μM), and nifedipine (3 μM) + MDL-12330A (100 μM), respectively, on increases in catecholamine output induced by PACAP were examined with the same protocol used for group 1.

Perfusate sampling. Perfusate was sampled before and during infusion of PACAP to determine catecholamine output. In all groups, the sampling during the basal state was performed for 60 s just before the stimulation, and the sampling during infusion of PACAP was performed for 200 s.

Determination of adrenal catecholamine output. As an internal standard, 25 ng of 3,4-dihydroxybenzylamine, contained in 50 μl of 0.1 M perchloric acid, were added to each chilled tube containing perfusate sample. Catecholamines in the perfusate sample were measured by high-performance liquid chromatography (pump, model LC-100; autoinjector, model CMA/200; column, Biophase ODS-IV 4.0-mm ID × 110 mm, Bioanalytical Systems) with electrochemical detection (model LC-4C, Bioanalytical Systems). The mobile phase (0.1 M tartaric acid-0.1 M sodium acetate, pH 3.2) was delivered to the column at a flow rate of 0.5 ml/min. The effluent was passed through an amperometric detector cell with a glassy carbon electrode and an Ag-AgCl reference electrode. The potential between the electrodes was kept constant at 700 mV. The amount of catecholamines was calculated from the area in the chromatogram on the basis of internal calibration methods. Epi and NE output (in ng/min) was calculated by multiplying perfusate catecholamine concentration (in ng/ml) by perfusion rate (0.2 ml/min). The PACAP-induced increases in catecholamine output were calculated by subtracting catecholamine output before the PACAP infusion from that during the PACAP infusion.

Analysis of data. Values are means ± SE. One-factor ANOVA was used for statistical analysis, and Dunnett’s test was applied for multiple comparison. Student's t-test was used to compare the values between two experimental periods. P < 0.05 was considered to be statistically significant.

Drugs. PACAP-27, ω-conotoxin GVIA, and ω-conotoxin MVIIIC were obtained from the Peptide Institute (Osaka, Japan); nifedipine and MDL-12330A hydrochloride were obtained from Sigma Chemical (St. Louis, MO). Nifedipine was dissolved in ethanol and diluted to the required concentration with Krebs-Henseleit solution under dim light immediately before use. MDL-12330A was dissolved in distilled water and diluted to the required concentrations with Krebs-Henseleit solution. Other drugs were dissolved in Krebs-Henseleit solution. Neither the ethanol- nor the distilled water-containing Krebs-Henseleit solution affected the PACAP-induced catecholamine output (data not shown).

RESULTS

Increases in catecholamine output in response to PACAP. Basal Epi and NE output from the adrenal gland before the first trial was 30.5 ± 2.5 and 5.3 ± 0.5 ng/min (n = 68), respectively, in all groups. Infusion of PACAP (100 nM) into the adrenal gland increased Epi and NE output in the first trial of each experimental
The PACAP-induced increase in Epi and NE output was 105.5 ± 6.1 and 13.7 ± 0.8 ng/min (n = 68), respectively, in all groups, and there were no significant differences in PACAP-induced catecholamine output among the experimental groups.

Effects of calcium-free solution, VDCC blockers, and MDL-12330A on PACAP-induced increases in catecholamine output. Perfusion with calcium-free solution significantly inhibited the PACAP-induced increases in Epi and NE output by 51.1 ± 7.4 and 51.4 ± 7.9%, respectively (group 1; Fig. 1). Nifedipine (1 and 3 μM) significantly inhibited the PACAP-induced increases in Epi and NE output (group 2; Fig. 2). Percent inhibition by 3 μM nifedipine of Epi and NE output responses was 46.2 ± 4.1 and 36.5 ± 4.6%, respectively. In contrast, 10 and 100 nM ω-conotoxin GVIA (group 3; Fig. 3A) or 100 and 1,000 nM ω-conotoxin MVIIC (group 4; Fig. 3B) did not affect the PACAP-induced increases in Epi and NE output. MDL-12330A (30 and 100 μM) inhibited the PACAP-induced increase in Epi output, but it did not affect the PACAP-induced increase in NE output (group 5; Fig. 4). Percent inhibition by 100 μM MDL-12330A of increases in Epi and NE output induced by PACAP was 52.3 ± 3.1 and 1.3 ± 12.3%, respectively.

Effects of single or combined treatment of nifedipine and MDL-12330A. Nifedipine (3 μM) significantly inhibited the PACAP-induced increases in Epi and NE output by 40.1 ± 3.7 and 41.4 ± 4.9%, respectively (group 6; Fig. 5). MDL-12330A (100 μM) inhibited the PACAP-induced increase in Epi output, but it did not affect the increase in NE output (group 7; Fig. 5). Percent inhibition by MDL-12330A of increases in Epi and NE output induced by PACAP was 20.8 ± 3.8 and −0.3 ± 6.6%, respectively. Treatment with 3 μM nifedipine + 100 μM MDL-12330A significantly inhibited the PACAP-induced increases in Epi and NE output.
extracellular space, also contributes to the PACAP-induced catecholamine secretion from intracellular storage sites, as well as the influx from intracellular free calcium by the mobilization from cells (28). It is therefore likely that the elevation of intracellular calcium stores in bovine adrenal medullary PACAP is reported to cause calcium release from intracellular free calcium (28). It is therefore likely that the elevation of intracellular calcium stores in bovine cultured adrenal chromaffin cells (20, 27) and the isolated perfused rat adrenal gland (12). Perfusion of the adrenal gland with calcium-free solution completely inhibited the inhibition of the PACAP-induced catecholamine output (data not shown). We also observed that perfusion with the calcium-free solution completely inhibited the increases in Epi and NE output from basal output levels in each control period. **P < 0.01 compared with corresponding values obtained with each drug alone (groups 6–8; Fig. 5).

**DISCUSSION**

Infusion of 100 nM PACAP into the isolated perfused rat adrenal gland increased Epi and NE output. This finding is consistent with previous observations obtained in bovine cultured adrenal chromaffin cells (20, 27) and the isolated perfused rat adrenal gland (12). Perfusion of the adrenal gland with calcium-free solution inhibited the PACAP-induced increases in Epi and NE output, indicating that extracellular calcium contributes to the PACAP-induced catecholamine secretion.

It has been reported that calcium-free medium almost abolishes the catecholamine secretion induced by PACAP in porcine (13) and rat (23) adrenal chromaffin cells. In this study, however, ~50% of the PACAP-induced output responses remained during the perfusion with calcium-free solution. This would raise a question whether the perfusion sufficiently reduced extracellular calcium. However, addition of 1 mM EGTA to calcium-free solution failed to cause further inhibition of the PACAP-induced catecholamine output (data not shown). We also observed that perfusion with the calcium-free solution completely inhibited the increases in Epi and NE output induced by a nicotinic agonist, 1,1-dimethyl-4-phenylpiperazinium, in preliminary experiments (data not shown). In addition, PACAP is reported to cause calcium release from intracellular calcium stores in bovine adrenal medullary cells (28). It is therefore likely that the elevation of intracellular free calcium by the mobilization from intracellular storage sites, as well as the influx from extracellular space, also contributes to the PACAP-induced secretion of Epi and NE from the rat adrenal gland.

Nifedipine (1–3 μM) inhibited the PACAP-induced increases in Epi and NE output. The degree of inhibition by 3 μM nifedipine was similar to that obtained with calcium-free solution. This result agrees with the observation that L-type VDCC blockers inhibit the catecholamine responses in porcine adrenal medullary cells (13), bovine cultured adrenal chromaffin cells (20), and dog adrenal medulla in vivo (9). However, the PACAP-induced catecholamine secretion from rat cultured adrenal chromaffin cells, which entirely depends on extracellular calcium, was reported to be resistant to blockade of L-type VDCCs (23). The difference in the participation of L-type VDCCs could be ascribed to experimental conditions: PACAP was infused for 3 min in our present study, whereas PACAP was applied for only 10 s in the study using rat cultured adrenal chromaffin cells (23). A study in the dog adrenal gland suggested that L-type VDCCs are involved in the sustained increases, rather than the initial increases, in catecholamine output induced by PACAP infusion (9), which may explain the difference. Thus L-type VDCCs may play an important role in the PACAP-induced secretion of Epi and NE from the rat adrenal gland.

Electrophysiological studies have suggested that L-type VDCCs predominate, but other subtypes of VDCCs, such as N-, P-, and Q-types, also exist in rat adrenal chromaffin cells (8). However, the contribution of these channels to the PACAP-induced Epi and NE secretion has not been examined in the rat adrenal gland. In the present study, ω-conotoxin GVIA (10 and 100 nM), an N-type VDCC blocker, or ω-conotoxin MVIIIC (100 and 1,000 nM), a P/Q-type VDCC blocker, did not affect the PACAP-induced increases in Epi and
NE output. The failure of these VDCC blockers to affect the catecholamine secretion responses may not be due to a poor tissue penetration ability or insufficient concentration, because ω-conotoxin GVIA (10 nM) and ω-conotoxin MVIIC (1,000 nM) attenuate neurally evoked catecholamine secretion in the perfused bovine adrenal gland (21) and in the perfused rat adrenal gland (19), respectively. Thus our results suggest that N-, P-, and Q-type VDCCs have no role in the PACAP-induced catecholamine secretion from the rat adrenal gland.

MDL-12330A (30 and 100 μM), an adenylyl cyclase inhibitor (11, 15), inhibited the increase in Epi, but not NE, output induced by PACAP. Although it is well known that PACAP activates adenylyl cyclase in rat anterior pituitary cells (17), it has not been fully understood whether activation of adenylyl cyclase is involved in the mechanism of PACAP-induced adrenal catecholamine secretion. The present study is the first to demonstrate effects of MDL-12330A on PACAP-induced secretion of catecholamines and to reveal the difference between its effects on Epi and NE secretion. Epi and NE are stored in and secreted from distinct chromaffin cells (3, 4). There may be dissociation between the mechanisms of catecholamine secretion from these two types of adrenal chromaffin cells. For example, potassium-evoked secretion of Epi and NE is preferentially controlled by Q- and L-type VDCCs, respectively, in the bovine cultured chromaffin cells (16), and NE, but not Epi, secretion mediated by the nicotinic receptor is modulated by small-conductance calcium-activated potassium channels in the rat adrenal gland (18). The mechanisms of adrenal catecholamine secretion induced by PACAP may be also different between Epi- and NE-containing cells. The results of this study suggest that activation of adenylyl cyclase is involved in the PACAP-induced Epi, but not NE, secretion from the rat adrenal gland.

The present study suggested that opening of L-type VDCCs and activation of adenylyl cyclase contribute to the PACAP-induced adrenal catecholamine secretion. Activation of adenylyl cyclase causes enhancement of cAMP production followed by activation of protein kinase A. Protein kinase A activates VDCCs of adrenal chromaffin cells (1, 2). However, it remains unclear whether the adenylyl cyclase-cAMP-protein kinase A pathway activated by PACAP causes opening of L-type VDCCs in the mechanism of PACAP-induced catecholamine secretion. Therefore, we examined their interaction by comparing the effects of treatment with 3 μM nifedipine + 100 μM MDL-12330A with that of a single treatment of each drug on the PACAP-induced adrenal catecholamine secretion. The Epi secretion response was inhibited by nifedipine + MDL-12330A, the effect of which was higher than that obtained with each drug alone. In these experiments (groups 7 and 8), inhibition by MDL-12330A was smaller than that observed in the above-mentioned experiments (group 5) in which MDL-12330A was applied consecutively at 30 μM and then at 100 μM. This difference would indicate that MDL-12330A causes a time-related action but may not affect the comparison between the results obtained with the single treatment and the combined treatment, because MDL-12330A was applied by the same protocol in these experiments. The NE secretion response was inhibited by single treatment with nifedipine, but not by single treatment with MDL-12330A, and the degree of inhibition by the combination of these drugs was similar to that obtained with nifedipine alone. These additive inhibitory effects by nifedipine + MDL-12330A at the highest concentrations suggest that opening of L-type VDCCs and activation of adenylyl cyclase are involved in the distinct pathways of the PACAP-induced catecholamine secretion from the rat adrenal gland.

In the present study, inhibition of calcium influx and/or adenylyl cyclase did not abolish the PACAP-induced catecholamine secretion. The PACAP-induced adrenal catecholamine secretion is suggested to result from activation of PAC1 receptors (13, 29). We also observed that a PAC1 receptor blocker, PACAP-(6–38) (24), completely inhibited the PACAP-induced catecholamine secretion from the rat adrenal gland (7). PAC1 receptors are known to couple with Gs-adenylate cyclase and Gα-phospholipase C-inositol trisphosphate pathways (26), and it is suggested that PACAP causes the calcium release from inositol trisphosphate stores in porcine adrenal medullary chromaffin cells (13) or from ryanodine/caffeine stores in bovine adrenal medullary cells (28). Therefore, PACAP may also cause calcium mobilization from the intracellular storage sites via these pathways, which are independent of opening of L-type VDCCs or activation of adenylyl cyclase, to induce catecholamine secretion in the rat adrenal gland. The process of PACAP-induced adrenal catecholamine secretion seems to involve multiple signaling pathways.

In conclusion, our results suggest that opening of L-type VDCCs is involved in the process of PACAP-induced secretion of Epi and NE, activation of adenylyl cyclase is involved in the process of PACAP-induced Epi, but not NE, secretion, and these two pathways work independently of each other in the rat adrenal gland.

Perspectives

The present study indicated the difference between participation of adenylyl cyclase in the PACAP-induced Epi and NE secretion. It is well known that hypoglycemia and hemorrhagic shock preferentially induce adrenal Epi and NE secretion, respectively (6, 14). Thus the adrenal gland can release each catecholamine in accordance with physiological demands. This preferential catecholamine secretion is controlled by the central nervous system, which may separately send nerve impulses to the Epi- and NE-containing cells (5). In addition, peripheral mechanisms that distinguish Epi and NE secretion may also exist within the adrenal gland. As described in the DISCUSSION, Lomax et al. (16) reported that Q- and L-type VDCCs participate in Epi and NE secretion, respectively, in bovine adrenal chro-
maffin cells. We recently reported that small-conduc-
tance calcium-activated potassium channels modulate
NE, but not Epi, secretion mediated by nicotinic recep-
tors in the rat adrenal gland (18). In this regard, the
difference in participation of adenylate cyclase found in
our present study could also play a role as one of the
above-mentioned peripheral mechanisms.

However, it remains unknown when and how these
peripheral mechanisms are activated to modulate each
catecholamine secretion, and there has been no direct
evidence for differences in ion channels and signal
transduction pathways between the Epi- and NE-con-
taining cells. Furthermore, little is known about the
contribution of endogenous PACAP to the adrenal cat-
tocholinergic secretion. The physiological significance
of these mechanisms remains to be further elucidated.

This work was supported in part by Ministry of Education, Sci-
ence and Culture, Japan, Grant for Scientific Research 10877371.

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