Time-Dependent Effects of the Neuropeptide PACAP on Catecholamine Secretion

Stimulation and Desensitization

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Abstract—Pituitary adenylyl cyclase-activating polypeptide (PACAP) is a potent endogenous secretagogue for chromaffin cells. We previously reported that PACAP coupled to the PAC1 receptor to evoke dihydropyridine-sensitive early (15 to 20 minutes) catecholamine secretion and cAMP response element binding protein–mediated trans-activation of the secretory protein chromogranin A promoter in PC12 pheochromocytoma cells. In this report, we studied whether the secretory and transcriptional responses elicited by PACAP were subject to desensitization. We found that PACAP evoked distinct immediate (0 to 20 minutes) and long-lasting (20 to 180 minutes) effects on catecholamine secretion. Initial secretory and chromogranin A trans-activation responses induced by PACAP were desensitized in a dose-dependent fashion after preexposure of cells to PACAP, and the IC50 doses of PACAP for desensitization were 8- to 32-fold lower than the EC50 activating doses for secretion or transcription. Desensitization of the initial secretory response was associated with decreased Ca2+ influx through L-type voltage-operated Ca2+ channels. Acute exposure to PACAP also triggered long-lasting (up to 3 hours), extracellular Ca2+-dependent, pertussis toxin–insensitive catecholamine secretion; indeed, even after short-term (20 minutes) exposure to PACAP and removal of the secretagogue, PC12 cells continued to secrete norepinephrine up to 76.9±0.22% of cellular norepinephrine content after 3 hours. A phospholipase C-β inhibitor (U-73122) blocked this extended secretory response, which was dependent on low-magnitude Ca2+ influx resistant to several L-, N-, P/Q-, or T-type Ca2+ channel antagonists, but sensitive to Zn2+, Ni2+, Cd2+, or to the store-operated Ca2+ channel blocker SKF96365. A less than additive effect of the sarcoplasmic reticulum Ca2+-ATPase inhibitor thapsigargin plus PACAP on this sustained secretion also supported a contribution of store-operated Ca2+ entry to the sustained secretory response. We propose that PACAP-evoked secretion and transcription are subject to homologous desensitization in PC12 cells; however, PACAP also induces long-lasting secretion, even under dose and time circumstances in which acute, dihydropyridine-sensitive secretion has been desensitized. Although initial secretion is mediated by an L-type voltage-operated Ca2+ channel, extended secretion may involve a store-operated Ca2+ channel that is activated through a Gq11/phospholipase C-β/phosphoinositide signaling pathway. (Hypertension. 1999;34:1152-1162.)

Key Words: PC12 ■ chromaffin ■ chromogranin ■ peptides ■ catecholamines ■ desensitization

Pituitary adenyl cyclase–activating polypeptide (PACAP) is a member of the secretin/glucagon/vasoactive intestinal peptide (VIP) regulatory peptide family.1,2 This neuropeptide occurs in both 27- and 38- (PACAP38) amino acid forms.1 PACAP exhibits a variety of biological activities including regulation of hormone production and secretion in endocrine organs such as the pituitary, thyroid, and pancreas.3,4 The adrenal gland is innervated by a dense network of PACAP-immunoreactive fibers, and abundant PACAP binding sites occur on the surface of chromaffin cells.5,6 In addition to the classic preganglionic neurotransmitter acetylcholine, the noncholinergic transmitter PACAP is likely to play an important role in governing both secretion and synthesis of catecholamines in chromaffin cells.7-10

We recently reported that PACAP triggers both catecholamine secretion and transcriptional activation of the regulated secretory protein chromogranin A in PC12 pheochromocytoma cells.11 Other studies have suggested distinct differences between PACAP actions and those of the nicotinic cholinergic agonist acetylcholine.8,12 In contrast to nicotine, whose secretory response in chromaffin cells shows rapid desensitization,13 chronic exposure of chromaffin cells to PACAP can evoke long-lasting Ca2+ influx7 and catecholamine secretion,12 without apparent desensitization of the secretory response.

PACAP/VIP receptors belong to the large family of the 7 transmembrane–spanning guanine nucleotide-binding G protein–coupled receptors (GPCRs). Three classes of PACAP/
VIP receptors (PAC1, VPAC1, VPAC2) have been identified and cloned. \(^{14,15}\) PAC1 receptors, which have high selectivity for PACAP but low affinity for VIP, exist as 6 splice-variant forms. \(^{15,16}\) PAC1 can couple to activation of both the adenylyl cyclase (AC) and the phospholipase C-β/phosphoinositide (PLC-β/PI) pathways, through \(G_i\) and \(G_{q/11}\) classes of \(G\) protein subunits. \(^{15,17}\) Sustained exposure of GPCRs to agonist generally induces desensitization of the receptor responsiveness. Rapid desensitization results from uncoupling of the heterotrimeric G protein from its receptor, as a consequence of receptor phosphorylation. \(^{18}\) To date, functional desensitization of the PAC1 receptor in chromaffin cells remains uncharacterized.

In PC12 cells, PACAP-induced catecholamine secretion and chromogranin A transcription are both mediated by PAC1. \(^{11}\) The present study reveals that both norepinephrine release and chromogranin A trans-activation, which are induced by PACAP, desensitize after limited preexposure of PC12 cells to very low doses of peptide (IC\(_{50}\) values 18- to 32-fold less than the corresponding EC\(_{50}\) values). Desensitization of secretion seems to be the consequence of diminished \(Ca^{2+}\) influx through L-type voltage-operated \(Ca^{2+}\) channels (VOCC). However, PACAP also triggers a potent, long-lasting, non-desensitizing catecholamine release, even under dose and time circumstances in which acute, dihydropyridine (DHP)-sensitive secretion has been desensitized. We propose that sustained catecholamine release is mediated by a \(G_{q/1}\) protein coupling PAC1 to the PLC-β/PI pathway in a process that is dependent on store-operated \(Ca^{2+}\) channels (SOCCs).

**Methods**

**Peptides and Reagents**

Synthetic PACAP38 and PACAP6-38 were from Peninsula Laboratories. Thapsigargin, bisindolylmaleimide, chelerythrine, calphostin C, flunarizine, bepridil, pertussis toxin (PTX), SKF96365, nifedipine, and caffeine were from Calbiochem Corporation. \(ZnCl_2\) was obtained from Aldrich Chemical. Nicotine, ETG, NiCl\(_2\), CdCl\(_2\), and ruthenium red were from Sigma Chemical Company. 2.5S NGF was from Life Technologies. U-73122, U-73343, and ryanodine were from Research Biochemicals Incorporated. \(\omega\)-Conotoxin GVIA was from Research Biochemicals Incorporated or Alexis Corporation; \(\omega\)-conotoxin MVIC was obtained from Alexis Corporation.

**Cell Culture**

Passage 8 PC12 rat pheochromocytoma cells, which were obtained from Dr David Schubert (Salk Institute, La Jolla, Calif), were cultured in high-glucose DMEM that was supplemented with 10% heat-inactivated horse serum and 5% heat-inactivated fetal bovine serum (Gemini Bioproducts), streptomycin (100 \(\mu\)g/mL), and penicillin (100 U/mL).

**Catecholamine Secretion**

Catecholamine secretion from PC12 cells was performed as previously described. \(^{18}\) Briefly, cells were grown on poly-\(\alpha\)-lysine–coated 6-well Falcon plates at 40% to 50% confluence. Cells were transfected with 2 \(\mu\)g of supercoiled plasmid DNA per well, with a polycation method (Superfect, Qiagen Inc). Three hours after transfection, PC12 cells were treated for 4 hours with the indicated concentrations of PACAP. Cells were harvested as previously described \(^{21}\) and assayed for luciferase reporter activity and protein concentration. Luciferase results were normalized to cell protein content.

**Statistics**

Values are given as the mean±1 SEM for triplicate determinations. Statistical analysis was performed by unpaired Student \(t\) test or by 2-way ANOVA. Differences were considered significant at \(P<0.05\).

**Results**

**PACAP-Evoked Catecholamine Secretion Is Subject to Desensitization**

PACAP triggered catecholamine secretion from PC12 cells in a concentration-dependent manner. \(^{11}\) Increased norepinephrine release became significant at \(\geq 1\) nmol/L peptide and reached a maximum at 1 \(\mu\)mol/L peptide, with EC\(_{50}\) \(\approx 23\) nmol/L (Figure 1A).

Previous exposure of cells to PACAP for 10 minutes, followed by washing (twice for 5 minutes), caused impairment of the subsequent stimulatory effect of 250 nmol/L PACAP on catecholamine secretion (Figure 1B). As illustrated in Figure 1B, desensitization of PACAP-induced secretion became significant when cells were preexposed to \(\geq 1\) nmol/L PACAP. Desensitization of subsequent secretion by prior PACAP reached a maximum at 20 to 50 nmol/L PACAP preincubation, with IC\(_{50}\) \(\approx 1.3\) nmol/L (Figure 1B). Of note, desensitization of the subsequent PACAP secretory process that is dependent on store-operated \(Ca^{2+}\) channels (VOCC). However, PACAP also triggers a potent, long-lasting, nondesensitizing catecholamine release, even under dose and time circumstances in which acute, dihydropyridine (DHP)-sensitive secretion has been desensitized. We propose that sustained catecholamine release is mediated by a \(G_{q/1}\) protein coupling PAC1 to the PLC-β/PI pathway in a process that is dependent on store-operated \(Ca^{2+}\) channels (SOCCs).
response was not observed when the wash step preceding rechallenge with PACAP was omitted (data not shown).

To exclude the possibility that the decreased secretory response observed after rechallenge with PACAP (Figure 1B) could reflect depletion of catecholamines in the intracellular (secretory granule) pool after preexposure to PACAP rather than true desensitization of the PACAP receptor, we examined the effect of PACAP preincubation on catecholamine secretion induced by direct membrane depolarization with 55 mmol/L KCl. Preincubation of PC12 cells with 20 nmol/L PACAP did not affect the secretory response to subsequent membrane depolarization with KCl: KCl caused 48.56 ± 0.27% secretion of norepinephrine, and this response was not altered by PACAP preexposure (49.01 ± 0.57% secretion). This result indicates that prior exposure of cells to a submaximal secretory dose of PACAP has little or no effect to deplete the intracellular pool of catecholamines in secretory granules.

Because Ca$^{2+}$ influx through L-type VOCC mediates PACAP-induced catecholamine secretion from chromaffin cells,7,8,11 we therefore investigated the effect of a desensitizing preincubation dose of PACAP (20 nmol/L) on Ca$^{2+}$ influx triggered by a subsequent stimulation with PACAP (250 nmol/L). As illustrated in Figure 2, 250 nmol/L PACAP triggered $^{45}$Ca$^{2+}$ uptake in PC12 cells to a value of 246 ± 18.5% over control (=100%). Preincubation of cells with a desensitizing concentration of peptide (20 nmol/L) resulted in a substantial decrement in the subsequent PACAP-induced $^{45}$Ca$^{2+}$ uptake (Figure 2). Under these conditions, $^{45}$Ca$^{2+}$ uptake declined to a value of 149 ± 5.3% over control, suggesting that desensitization of the secretory response occurred at a step proximal to Ca$^{2+}$ influx through L-type VOCC.

**PACAP Trans-Activation of Chromogranin A Transcription Is Subject to Desensitization**

PACAP trans-activates a transfected chromogranin A promoter in PC12 cells in a concentration-dependent manner (Figure 3A and Reference 11). The activity of a transfected 1200-bp chromogranin A promoter/luciferase reporter plasmid was increased significantly at 20 hours of exposure to 0.1 nmol/L PACAP, with EC$^{50}$ of 10 nmol/L (Figure 3A). By contrast, preexposure of cells to PACAP, followed by washing, inhibited in a dose-dependent manner the subsequent stimulatory effect of 250 nmol/L PACAP on transcriptional activation of the chromogranin A promoter (Figure 3B). Desensitization of PACAP-induced transcription became significant when cells were preexposed to 0.1 nmol/L PACAP, and complete desensitization of transcription was reached after a 10 to 50 nmol/L PACAP preincubation (IC$^{50}$ of 0.31 nmol/L, Figure 3B).

**PACAP-Evoked Catecholamine Secretion Includes a Nondesensitizing Component That Is Biphasic and Long Lasting: Effect of Acute Stimulation of PC12 Cells With PACAP or Nicotine on Catecholamine Secretion**

Chronic exposure of bovine chromaffin cells to PACAP elicits long-lasting catecholamine secretion for up to several...
hours without apparent desensitization. 12 We therefore questioned whether acute exposure of PC12 cells to PACAP could provoke such a sustained secretory response. Indeed, short-term (≤20 minutes) exposure of PC12 cells to 250 nmol/L PACAP elicited long-lasting catecholamine release for up to 3 hours (Figure 4A). Thereafter, even replacement of the PACAP-containing medium by PACAP-free medium did not impair this sustained PACAP-evoked catecholamine secretion. For example, after 1 hour of exposure to PACAP-depleted medium (ie, time segment 80 to 100 minutes), the magnitude of net norepinephrine release was similar to that measured after 20 minutes of acute exposure to PACAP. At time segment 160 to 180 minutes, norepinephrine secretion was still maintained at a value of 7.9±0.14% over basal. When cumulative catecholamine release was measured for 3 hours after an acute exposure of cells (20 minutes) to PACAP, a remarkable 76.9±0.22% of total cell content of catecholamines had been secreted (see Figure 4 legend for details of calculation). In sharp contrast, acute treatment of PC12 cells with 60 μmol/L nicotine induced massive (24% net) catecholamine secretion within 20 minutes followed by a rapid decay of the response that returned to a basal secretion value within 40 to 60 minutes (Figure 4B).

Effect of Blockade of the Initial Phase of Catecholamine Secretion on PACAP-Induced Prolonged Secretory Response

Catecholamine release triggered by PACAP from chromaffin cells is dependent on the presence of Ca2+ in the extracellular...
medium. To determine whether blockade of the initial phase of the secretory response can prevent PACAP from further evoking the prolonged catecholamine release, we examined the effect of Ca\(^{2+}\) depletion during the first 20-minute stimulation period with 250 nmol/L PACAP. Consistent with previous studies, Ca\(^{2+}\)-deficient medium strongly impaired the secretory response triggered by PACAP within the acute, 0- to 20-minute time segment (Figure 5A). When Ca\(^{2+}\) was then reintroduced together with PACAP-free secretion medium, catecholamine release resumed for cells that had been acutely exposed to PACAP and reached secretion values in the same magnitude as that observed for cells that were not deprived of Ca\(^{2+}\) during the limited (20 minutes) exposure to PACAP (Figure 5A).

Effect of the PAC1 Antagonist PACAP6-38 on PACAP-Evoked Extended Catecholamine Secretion

In PC12 cells, the initial phase of catecholamine secretion that results from short-term exposure to PACAP (20 minutes) is inhibited by the PACAP fragment PACAP6-38, a potent competitive inhibitor of PAC1. To assess the contribution of the PAC1 receptor to PACAP-induced long-lasting catecholamine secretion, we examined the effect of continuous exposure of cells to PACAP6-38 (5 μmol/L). After a 20-minute incubation, cells were harvested for norepinephrine secretion determination. A and B, top: Effect of nifedipine or U-73122 on extended catecholamine secretion. A and B, bottom: Effect of nifedipine or U-73122 on extended catecholamine secretion. [3H]-L-norepinephrine–prelabeled PC12 cells were exposed to mock or 250 nmol/L PACAP. After a 20-minute incubation and at the indicated time segments, extracellular media were collected and exchanged for mock medium (A and B) or exposed to 5 μmol/L PACAP6-38 (B). Catecholamine secretion was evaluated as described in the legend of Figure 4.

Figure 5. Effect of blockade of PACAP-induced initial catecholamine secretion or PAC1 competitive antagonist PACAP6-38 on long-lasting secretory response from PC12 cells. [3H]-L-norepinephrine–prelabeled PC12 cells were exposed for 20 minutes to either secretion medium (mock) or Ca\(^{2+}\)-free secretion medium supplemented or unsupplemented with 250 nmol/L PACAP38. After a 20-minute incubation and at the indicated time segments, extracellular media were collected and exchanged for mock medium (A and B) or exposed to 5 μmol/L PACAP6-38 (B). Catecholamine secretion at each time segment was evaluated as described in the legend of Figure 4.

Figure 6. Effect of L-type VOCC blocker nifedipine or PLC-β inhibitor U-73122 on PACAP-induced initial and long-lasting catecholamine secretion from PC12 cells. A and B, top: Effect of nifedipine or U-73122 on catecholamine secretion over the initial 20 minutes. [3H]-L-norepinephrine–prelabeled PC12 cells were incubated in mock medium, 250 nmol/L PACAP, 250 nmol/L PACAP plus 10 μmol/L nifedipine (A, top), or indicated concentration of U-73122 (B, top). After a 20-minute incubation, cells were harvested for norepinephrine secretion determination. A and B, bottom: Effect of nifedipine or U-73122 on extended catecholamine secretion. [3H]-L-norepinephrine–prelabeled PC12 cells were exposed to mock or 250 nmol/L PACAP. After a 20-minute incubation and at the indicated time segments, extracellular media were collected and exchanged for mock medium or secretion medium plus 10 μmol/L nifedipine (A, bottom) or 1 μmol/L U-73122 (B, bottom). Catecholamine secretion was evaluated as described in the legend of Figure 4.
a 20-minute exposure of cells to 250 nmol/L PACAP alone, continuous application of 10 μmol/L nifedipine over a period of up to 100 minutes did not influence the extended effect of PACAP on catecholamine secretion, suggesting that the prolonged secretory response triggered by PACAP is not mediated by a DHP-sensitive Ca\(^{2+}\) channel, ie, the L-type VOCC (Figure 6A), which contrasts with the initial phase secretion.\(^{11}\)

**Effect of PTX or the PLC-β Inhibitor U73122 on PACAP-Induced Initial and Extended Catecholamine Secretion**

PAC1 not only stimulates the effector AC but also transduces its signal through the PLC-β/IP pathway in PC12 and other cell types.\(^{1,15,24}\) Monomeric α subunits of the G\(_i/o\) family of G proteins as well as βγ heterodimeric subunits of the G\(_i/o\) class mediate activation of PLC-β.\(^{25}\) These 2 families of G proteins can be distinguished by their sensitivity to PTX: G proteins of the G\(_i/o\) family are sensitive to ADP-ribosylation by the toxin, whereas the G\(_o\) family is PTX-insensitive. To probe the type of G protein involved in both initial and long-term secretory responses stimulated by PACAP, we examined the effect of a pretreatment with PTX. Preexposure of PC12 cells to PTX (16 hours, 100 ng/mL) did not modify either initial or extended secretion evoked by 250 nmol/L PACAP (data not shown). Continuous exposure of cells to U-73122 (1 μmol/L), a selective antagonist of PLCs, after 20 minutes of stimulation with PACAP, impaired the prolonged secretory response triggered by PACAP without affecting the initial phase of the secretory response (Figure 6B). These findings provide evidence that both initial and long-term secretory responses triggered by PACAP in PC12 cells are mediated by a PTX-insensitive G protein, whereas only the prolonged secretory response involves a PLC-β-mediated pathway. In contrast, the inactive isomer U-73343 (negative control) did not affect either acute or sustained PACAP-triggered catecholamine release (data not shown).

**PACAP Requires Extracellular Ca\(^{2+}\) Influx for its Prolonged Effect on Catecholamine Secretion**

To determine the contribution of Ca\(^{2+}\) to PACAP-induced long-term secretion, we examined the influence of a Ca\(^{2+}\)-deficient environment and the effect of Zn\(^{2+}\), a nonselective competitive inhibitor of a variety of cell surface Ca\(^{2+}\) channels. After 20 minutes of PACAP exposure, switching to a Ca\(^{2+}\)-free medium greatly impaired the long-lasting secretion induced by 250 nmol/L PACAP (Figure 7A). Under these conditions, catecholamine release was completely abolished as early as 40 minutes after Ca\(^{2+}\) removal. The inorganic cation Zn\(^{2+}\) (100 μmol/L) completely inhibited initial secretion (Reference 11 and Figure 7A) and also inhibited the long-lasting secretion induced by 250 nmol/L PACAP by ~65% to 85% (Figure 7A). Hence, these results suggest that the influx of extracellular Ca\(^{2+}\) through cell surface channels is essential for the prolonged effect of PACAP on the secretory response. However, Ca\(^{2+}\) uptake measurement after short-term exposure to 250 nmol/L PACAP revealed a continuing, modest influx of extracellular Ca\(^{2+}\) (Figure 7B). After the initial burst of Ca\(^{2+}\) influx (t\(_0\)→t\(_{2\text{ minutes}}\), Figures 2 and 7B), long-term Ca\(^{2+}\) uptake measured during 2-minute periods of sequential 20-minute time segments was modestly, though consistently, greater than that measured...
under basal conditions (Figure 7B). These results suggest that a low-magnitude influx of extracellular Ca\(^{2+}\), after the initial massive Ca\(^{2+}\) influx, is at least necessary (though perhaps not sufficient) for PACAP-induced long-lasting catecholamine secretion.

Characterization of the Role of Specific Cell Surface VOCC Subtypes (N-, P-, Q-, and T-type) in Long-lasting Catecholamine Secretion Induced by PACAP

Cell membrane VOCCs have been classified physiologically or pharmacologically as L, N, P/Q, T, or R types, and most of these channels (eg, L, N, and P/Q types) have been detected in PC12 cells. Because the L-type VOCC inhibitor nifedipine did not impair PACAP-induced extended secretion (Figure 6A), we investigated the role of non-L-type Ca\(^{2+}\) channels in this sustained secretion. We first tested the effect of a combination of 1 \(\mu\)mol/L \(\omega\)-conotoxin GVIA (a highly selective N-type VOCC antagonist) with 1 \(\mu\)mol/L \(\omega\)-conotoxin MIVIC (an N- and P/Q-type VOCC blocker). Long-lasting catecholamine release was unaltered by the combination of these 2 \(\omega\)-conopeptides at up to 100 minutes (data not shown).

To further examine potential contributions of DHP-insensitive Ca\(^{2+}\) channels to the prolonged secretory effect of PACAP, we used nerve growth factor (NGF; 100 ng/mL, 7 days) to differentiate the PC12 cell line. PC12 cells differentiated by NGF express both L- and N-type channels but show a preferential increase in the N-type on differentiation. As in undifferentiated PC12 cells, short-term (20 minutes) exposure of NGF-treated PC12 cells to 250 nmol/L PACAP elicited long-lasting catecholamine release (data not shown). However, continuous treatment with the N-type VOCC blocker \(\omega\)-conotoxin GVIA (5 \(\mu\)mol/L) did not diminish the secretory response for up to 100 minutes (data not shown), suggesting that N-type Ca\(^{2+}\) channels may not contribute to the prolonged effect of PACAP.

Finally, exposure of PC12 cells to the chemical VOCC inhibitors bepridil, a T- and L-type Ca\(^{2+}\) channel blocker, and flunarizine, which selectively blocks T-type Ca\(^{2+}\) channels, failed to antagonize PACAP-induced long-lasting secretion (data not shown). Another way to distinguish VOCCs is to compare the relative blocking effects of the inorganic divalent cations Ni\(^{2+}\) and Cd\(^{2+}\). For example, Ni\(^{2+}\) is more potent T-type blocker than Cd\(^{2+}\), whereas Cd\(^{2+}\) is more effective than Ni\(^{2+}\) in blocking N- and L-type channels. After the cells were stimulated for 20 minutes with 250 nmol/L PACAP, both Ni\(^{2+}\) and Cd\(^{2+}\) exhibited dose-dependent inhibition of norepinephrine release for up to 100 minutes, with >70% inhibition at 250 \(\mu\)mol/L divalent cation (data not shown). However, we did not observe any difference in the potency of these inorganic divalent cations to reduce the secretory response. The IC\(_{50}\) for Ni\(^{2+}\) (average IC\(_{50}\) value = 9.14±3.3 \(\mu\)mol/L) were not significantly different (P>0.05) from those measured when cells were incubated with Cd\(^{2+}\) (average IC\(_{50}\) value = 5.10±1.2 \(\mu\)mol/L).

Contribution of SOCCs to Long-Lasting Catecholamine Secretion Triggered by PACAP

Store-operated Ca\(^{2+}\) entry (also referred as capacitative Ca\(^{2+}\) entry) from the extracellular space is a widespread mecha-
only little desensitization for a period of up to 100 minutes. Coapplication of thapsigargin with PACAP (250 nmol/L) caused additive stimulation of secretion within the initial (0 to 20 minutes) phase. Thereafter, by 40 to 100 additional minutes in PACAP-free secretion medium, the secretory response in cells treated with initial (20 minutes) PACAP plus sustained (0 to 100 minutes) thapsigargin was no greater than in cells treated with initial PACAP alone (Figure 8A).

Taken together, our data suggest that PACAP and thapsigargin use distinct Ca\textsuperscript{2+} entry pathways to elicit the initial phase (≤20 minutes) of catecholamine release, while the less than additive effect of combined PACAP and thapsigargin during the prolonged phase of secretion suggests that the 2 compounds may share the same signaling pathway, i.e., Ca\textsuperscript{2+} release from intracellular stores, followed by activation of capacitative Ca\textsuperscript{2+} entry. To further probe a possible contribution of SOCCs to long-lasting catecholamine secretion evoked by PACAP, we tested the effect of the imidazole derivative SKF96365, a recently characterized SOCC/non-VGCC blocker.\textsuperscript{32,36} After the cells were stimulated for 20 minutes with 250 nmol/L PACAP, SKF96365 (50 μmol/L) markedly inhibited subsequent norepinephrine release for up to 100 minutes (Figure 8B), supporting the involvement of a store-operated Ca\textsuperscript{2+} entry mechanism in PACAP-induced extended secretion.

**Contribution of Ryanodine/Caffeine to Long-Lasting Catecholamine Secretion Triggered by PACAP**

Incubation of PC12 cells with 40 mmol/L caffeine, which activates Ca\textsuperscript{2+} release from ryanodine-sensitive stores,\textsuperscript{37} did not affect the PACAP-induced extended secretory response (data not shown). Similar results were obtained when Ca\textsuperscript{2+} flux through RyRs was antagonized by the polycationic dye ruthenium red (50 μmol/L) or when RyRs were locked in an open state by a low dose (10 μmol/L) of the irreversible RyR inhibitor ryanodine (data not shown).

**Discussion**

**PACAP Signaling**

PACAP is emerging as a potent peptidergic neurotransmitter that governs both secretory and biosynthetic activities of sympatoadrenal cells.\textsuperscript{7–10} We previously stated\textsuperscript{11} that PACAP uses divergent signaling pathways in PC12 cells to evoke catecholamine secretion versus transcriptional activation of chromogranin A, a key component of secretory granules of neurons and neuroendocrine cells. PACAP signals to chromogranin A transcription through the proximal CRE response element (CRE) in cis and through cAMP, protein kinase A (PKA), and the transcription factor CRE binding protein (CREB) in trans. By contrast, a pathway that involves cytosolic Ca\textsuperscript{2+} entry through L-type voltage-operated channels is required for PACAP to stimulate catecholamine secretion. Both secretory and transcriptional responses are mediated by the PAC1 receptor in PC12 cells.\textsuperscript{11} Indeed, the “hop” RNA-splicing variant of PAC1, as defined by Spengler et al.,\textsuperscript{15} which couples to both AC and PLC-β, is expressed in chromaffin cells.\textsuperscript{38}

**Desensitization**

Prolonged or repeated stimulation of GPCRs results in desensitization of the cell response to further or repeated stimulation. Remarkably, however, sustained exposure of chromaffin cells to PACAP can also induce long-lasting Ca\textsuperscript{2+} influx\textsuperscript{7} and catecholamine secretion, phenomena apparently at odds with desensitization.\textsuperscript{12} We show here that PACAP-induced catecholamine secretion in PC12 cells is subject to rapid homologous desensitization, which does not result from depletion of the releasable intracellular catecholamine pool in secretory granules. Desensitization of the secretory response (IC50 ≈1.3 nmol/L) occurred with ≈18-fold less PACAP than that required to induce secretion (EC50 ≈23 nmol/L), suggesting that the signaling pathway that underlies PACAP-induced desensitization might be distinct from that mobilized during secretion. In other cell types, PACAP dissociates slowly (t1/2 >20 minutes) from its receptor\textsuperscript{23}; consequently, long-lasting binding of the peptide to its receptor may underlie subsequent homologous desensitization in PC12 cells. Surprisingly, we noticed that homologous desensitization of the secretory response occurred only when preincubation of the cells with PACAP was followed by repeated washing before rechallenge with the polypeptide. After washing, partial dissociation of PACAP from its binding site might be a necessary step to achieve full desensitization downregulation of PAC1, perhaps as a consequence of incompletely characterized changes in receptor conformation. As revealed by a Ca\textsuperscript{2+}-uptake experiment, homologous desensitization results in a diminution of PACAP-triggered extracellular Ca\textsuperscript{2+} influx (Figure 2). Previous studies by us and others\textsuperscript{7,8,11} indicate that Ca\textsuperscript{2+} influx through L-type channels is an essential requirement for PACAP secretory action.

PACAP-triggered trans-activation of the CRE-containing chromogranin A promoter was also subject to marked homologous desensitization (Figure 3). After limited preincubation of cells with PACAP, trans-activation of the chromogranin A promoter evoked by subsequent rechallenge with PACAP was inhibited (Figure 3). Moreover, the inhibitory effect of PACAPs (10 nmol/L) 3 hour preincubation on chromogranin A promoter expression was prolonged and could still be detected after 24 hours of subsequent high-dose (250 nmol/L) PACAP (data not shown). Although we have not directly examined chromogranin A transcription, our previous findings on activation of transfected chromogranin A promoter/luciferase reporter plasmids (Reference 11 and Figure 3) provide results consistent with the responses of the endogenous chromogranin A gene at the levels of both steady-state mRNA accumulation\textsuperscript{11,21,39,40} and initiation of new transcripts.\textsuperscript{21,39} Muller et al.\textsuperscript{41} reported that pretreatment of catecholaminergic neuron-like CATH.a cells with PACAP enhanced subsequent PACAP-induced CREB-mediated transcription, a finding in sharp contrast with our current results when considering that the chromogranin A promoter’s CRE domain is sufficient to account for its PACAP stimulation.\textsuperscript{11} The discrepancy between our findings and those of Muller et al.\textsuperscript{41} may reflect differential cAMP responses in PC12 cells versus mouse central nervous system catecholaminergic CATH.a cells. In PC12 cells, PACAP signals to chromogranin A transcription through the cAMP response...
Sustained Secretion

Because the secretory response to PACAP is subject to homologous desensitization after a 10-minute preincubation (Figure 1), the long-lasting (20 to 180 minutes) PACAP secretory response (Figure 4) is an initially unexpected characteristic of the PC12 cell secretory response to PACAP. The mechanism of PACAP-induced catecholamine secretion is far from being fully understood. Some studies suggest that cAMP mediates the stimulatory effect of PACAP, whereas others report that VOCCs play a key role in secretion. Thus, the signaling pathway that underlies the action of PACAP on catecholamine secretion remains elusive. Our study reveals that PACAP exhibits dual secretory effects on PC12 cells, which can be discriminated by their sensitivity to nifedipine (Figure 6A): the initial phase of the secretory response may be defined as a nifedipine-sensitive response, whereas the long-term response is characterized by its insensitivity to DHP. Extracellular Ca entry through cell surface Ca channels is a key event in PACAP’s sustained stimulation of catecholamine release (Figure 7A). However, although the initial secretory event triggered by PACAP requires a massive influx of external Ca likely through L-type VOCCs (Figure 2), a lower magnitude Ca entry through DHP-insensitive Ca channels seems required to maintain long-lasting catecholamine release (Figure 7B).

PACAP-induced long-term secretion in undifferentiated PC12 cells was completely resistant to L-, N-, P/Q-, and T-type channel blockers. Moreover, even a transition to PC12 cells was completely resistant to L-, N-, P/Q-, and Ca channel blockers. Babinski et al have shown cosecretion of leucine-enkephalin along with catecholamines from chromaffin cells in response to PACAP.

The PAC1 receptor can couple to activation of both AC and PLC-β, presumably through activation of G, G, and G proteins. However, the nature of the G protein involved in PACAP-stimulated catecholamine secretion remains unclear. The mechanism whereby a G-protein coupled agonist depolarizes the cell membrane can involve either direct or indirect coupling of the G-protein to an ion channel. We found that neither initial (20 minutes) nor extended (20 to 180 minutes) secretion after PACAP were mediated by PTX-insensitive G proteins, which effectively ruled out G, G, or G isoforms (data not shown). However, the signaling pathways whereby PACAP evokes its short-term versus extended secretory effects seem to be quite divergent. Typically, hydrolysis of phosphatidylinositol 4,5-bisphosphate by PLC-β generates IP, which releases Ca from intracellular stores, and 1,2-diacylglycerol (DAG), which activates protein kinase C (PKC). In chromaffin cells, activation of PKC enhances catecholamine secretion. However, the resistance of the initial catecholamine release to the PLC-β inhibitor U-73122 (Figure 6B) leads us to exclude a contribution of the IP/DAG/PKC pathway in initial secretion. Similarly, PACAP-induced initial secretion was not affected by chronic PKC inactivation nor by highly selective PKC inhibitors, such as bisindolylmaleimide, chelerythrine, or calphostin C (unpublished data). Finally, the additivity of thapsigargin plus PACAP on acute stimulation of catecholamine release (Figure 9), together with previous observations excluding a role of caffeine/ryanodine-sensitive stores to PACAP-induced initial secretion, argue against a contribution of intracellular Ca release to the initial secretory response after PACAP. One hypothetical mechanism for initial secretion is direct opening of L-type Ca channels by the G-protein Go, or by subunits released by GPCR activation. PKA might also activate Ca channels although we found no effect of the cAMP pathway on catecholamine secretion from PC12 cells.

In contrast to initial secretion, inhibition of sustained catecholamine release by U-73122 suggests involvement of a G/PLC-βI signaling pathway (Figure 6B). A recent study by Bennett et al suggests that both RyR and IP activation may promote SOCC-mediated Ca entry in PC12 cells. However, the contribution of capacitative Ca entry to catecholamine release from chromaffin cells remains unsettled. The lack of requirement of ryanodine/caffeine-sensitive stores for sustained secretion induced by PACAP (see Results) argues against participation of these stores in long-term catecholamine release. On the other hand, the less than additive stimulatory effect of combined PACAP and SERCA inhibition on the prolonged secretion (Figure 8A), together with the inhibitory effect of the SOCC (non-VOCC) blocker SKF96365 (Figure 8B), suggests that PACAP-mediated PLC-β activation may trigger Ca release from IP-sensitive intracellular stores, eventuating in the subsequent capacitative (SOCC) Ca influx. Direct activation of non-VOCCs by a G may also be considered as a mechanism that underlies prolonged secretion. In support of this viewpoint, the recently characterized family of Drosophila non-VOCC transient receptor potential and transient...
receptor potential–like channels suggest a direct activation of the transient receptor potential–like channel by G_{q11}.28 Indeed, the transient receptor potential and transient receptor potential–like channels may represent the insect homologues of the mammalian SOCC channels.49

Conclusions
This work supports the hypothesis that PACAP is a potent noncholinergic neurotransmitter that governs both secretory and biosynthetic activities of sympathoadrenal cells through a signaling pathway distinct from that used by the more classical aminegenic agonist acetylcholine. In conclusion, on the basis of our previous report11 and on the current results, we present a model (Figure 9) that depicts putative signal transduction pathways underlying time-dependent PACAP-induced catecholamine release and chromogranin A transcription in PC12 cells. Both the secretory and the transcriptional responses induced by PACAP are subject to desensitization. Desensitization of the secretory response is associated with decreased Ca^{2+} influx through L-type VOCCs, suggesting a site of desensitization proximal to this channel. Concomitantly, PACAP effectively triggers prolonged catecholamine secretion that may be dependent on (1) slow dissociation of PACAP from its receptor, (2) extracellular Ca^{2+} influx of low magnitude, and (3) mediation by a G_{q11} protein. The pharmacology of the prolonged secretory response was characterized by sensitivity to broad-spectrum Ca^{2+}-channel blockade (Zn^{2+} / Ni^{2+} / Cd^{2+}), resistance to L-, N-, P/Q-, and T-type blockers, sensitivity to PLC-β, and SOCC blockade. These findings, together with the less than additive effect of thapsigargin on PACAP-sustained secretion, strongly suggest that a store-operated Ca^{2+} entry mechanism underlies the extended effect of PACAP on catecholamine release. Further characterization of PACAP signaling pathways will require definitive identification of the SOCC channel involved in the sustained catecholamine release; possible candidates include the mammalian homologues of the *Drosophila* transient receptor potential and transient receptor potential–like Ca^{2+} entry channels.49

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References


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