A Non-cholinergic Transmitter, Pituitary Adenylate Cyclase-activating Polypeptide, Utilizes a Novel Mechanism to Evoke Catecholamine Secretion in Rat Adrenal Chromaffin Cells*

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Pituitary adenylate cyclase-activating polypeptide (PACAP) is the most potent non-cholinergic neurotransmitter to stimulate catecholamine secretion from rat chromaffin cells; however, the mechanism of action is not clear. We used amperometric detection of exocytosis and indo-1 monitoring of [Ca\(^{2+}\)] to identify PACAP actions in cultured chromaffin cells. PACAP (100 nM) required external Ca\(^{2+}\) to evoke secretion. However, unlike nicotine and KCl which caused immediate and relatively brief secretion, PACAP had a latency of 6.8 ± 0.96 s to the first secretory response and secretion continued for up to 2 min. PACAP elevation of [Ca\(^{2+}\)] showed similar latency and often remained above base line for several minutes following a brief exposure. ZnCl\(_2\) (100 \(\mu\)M) selectively inhibited PACAP-stimulated secretion and [Ca\(^{2+}\)], with little effect on nicotine-evoked responses. Nifedipine (10 \(\mu\)M) had little effect on PACAP-evoked secretion but inhibited nicotine-evoked secretion by more than 80%, while ω-conotoxin (100 nM) failed to affect either agonist. PACAP-stimulated cAMP levels required 5 s to significantly increase, consistent with the latency of exocytotic and Ca\(^{2+}\) responses. Forskolin (10 \(\mu\)M) caused responses similar to PACAP. PACAP-evoked exocytosis was blocked by the protein kinase A inhibitor adenosine 3',5'-cyclic monophosphothioate R\(_\text{-}\)diastereomer (R\(_\text{-}\)cAMPS). These data show that PACAP stimulates exocytosis by a mechanism distinctly different from cholinergic transmitters that appear to involve cAMP-mediated Ca\(^{2+}\) influx. Differences in receptor coupling mechanisms and pharmacology of Ca\(^{2+}\) entry stimulated by cholinergic and peptidergic agonists support the idea that the peptidergic system maintains catecholamine secretion under conditions where the cholinergic system desensitizes or otherwise fails.

There is convincing evidence that secretion of adrenal medullary hormones in various species is regulated by peptidergic neurotransmitters. Among a long list of peptides that exist in the adrenal medulla, vasoactive intestinal polypeptide (VIP)\(^1\) satisfies several important criteria to be classified as a non-cholinergic neurotransmitter in the rat adrenal gland (1). VIP-like immunoreactivity has been detected in nerve terminals of adrenal glands of several species (1–4). However, there is also evidence against the existence of VIP immunoreactive fibers in adrenal medulla (5) and splanchic neurons (6). While VIP stimulates the cAMP as well as phosphatidylinositol pathways (7), it is difficult to draw firm conclusions about the mechanism of peptide-evoked catecholamine secretion from studies of intact adrenal gland.

At the same time that VIP was being characterized as a medullary neurotransmitter, a new member of the VIP/glucagon/secretin family of peptides (pituitary adenylate cyclase-activating polypeptide, PACAP) was discovered and shown to be a most potent activator of adenyl cyclase (8). PACAP occurs in both a 27 (PACAP\(_{27}\)) and 38 (PACAP\(_{38}\)) amino acid form and has about 70% homology with VIP (8, 9). PACAP immunoreactive fibers have been identified in the adrenal gland\(^2\) (but see Ref. 10), and PACAP receptors have been demonstrated on chromaffin cells (10, 11). Release of PACAP-like material from perfused adrenal following splanchnic nerve stimulation has been demonstrated (12). Importantly, PACAP is one of the most potent secretagogues in chromaffin cells in culture (13–15) and in perfused adrenal gland (16). Finally, the adrenal gland has been reported to contain the second highest concentration of PACAP among peripheral organs of the rat (17). Based on these findings, we have suggested that both VIP and PACAP might function as non-cholinergic neurotransmitters controlling catecholamine synthesis and secretion in the rat adrenal medulla (Ref. 12 and see also Refs. 18 and 19). The secretory properties of PACAP have been examined in whole adrenal gland (16, 20) and mass cultures of chromaffin cells (13, 14); however, the mechanism of PACAP-evoked catecholamine secretion remains unclear.

We undertook the present work to identify the mechanism by which PACAP mobilizes Ca\(^{2+}\) and stimulates secretion in primary cultures of rat chromaffin cells. Amperometric detection of exocytosis and indo-1 fluorescence measurement of [Ca\(^{2+}\)], were used to define PACAP effects on single chromaffin cells. Our recently developed protocols were used to isolate and identify internal versus external sources of Ca\(^{2+}\) used in exocytosis. PACAP-evoked exocytosis was monitored in cells during transient exposure to a Ca\(^{2+}\)-free environment and in cells in the presence of Ca\(^{2+}\) but depleted of internal Ca\(^{2+}\) stores. Our findings show that a peptidergic transmitter utilizes extracellular Ca\(^{2+}\) to evoke catecholamine secretion, but by a mechanism distinctly different from acetylcholine acting on nicotinic and muscarinic receptors in this preparation. The data indicate that PACAP increases Ca\(^{2+}\) influx in association with cAMP production.

\(^1\) The abbreviations used are: VIP, vasoactive intestinal polypeptide; PACAP, pituitary adenylate cyclase-activating polypeptide.

\(^2\) X. Guo, A. R. Wakade, and R. Pourcho, unpublished results.
Catecholamine Secretion by PACAP

MATERIALS AND METHODS

Primary Cultures of Rat Chromaffin Cells—Chromaffin cells were cultured from 19- to 31-day-old Sprague-Dawley rat pups as described (21–23). Briefly, medullary fragments were incubated in 15 mg/ml type I collagenase and 0.5 mg/ml DNase ( Worthington) in phosphate-buffered saline, followed by trypsin (1.25 mg/ml in PBS) and DNase (0.5 mg/ml), and cells dissociated by trituration. Cells were washed by centrifugation (600 x g, 5 min) and plated on collagen-coated glass coverslips (M-199 medium (Life Technologies, Inc.), plus serum and 0.1 mM dexamethasone and used after 2–7 days. Immunohistochemical staining showed that cultures contained greater than 90% tyrosine hydroxylase positive cells.3 Coverslips with attached chromaffin cells were transferred to HEPES-buffered bath solution for monitoring of exocytosis or [Ca2+]i. The solution contained (in mM) 119 NaCl, 4.7 KCl, 1.2 MgSO4, 2.5 CaCl2, 10 HEPES, 10 glucose, pH 7.4, with NaOH. Ca2+-free solution had no added CaCl2 and was supplemented with 1 mM EGTA.

Electrochemical Detection of Exocytosis—Exocytosis from single rat chromaffin cells was detected using established micro-electrochemical techniques (15, 24–26). Eight-μm carbon fibers were cannulated into polyethylene tubing, pulled and cut (final tip diameter < 9 μm), and sealed into glass capillaries. Capillaries were back-filled with 3 mM KCl and a Ag/AgCl wire used for connection to an E1 400 potentiostat (holding potential: 650 mV) coupled to a personal computer for data acquisition and analysis as described (15). Fiber carbon electrodes were positioned within 1 μm of a chromaffin cell to record currents resulting from oxidation of exocytically released catecholamines (25, 26).

Control of Internal and External Ca2+—Secretagogues in control or Ca2+-free bath solutions were applied by pressure ejection from pulled glass capillaries (–10 μm tip diameter) positioned about 20 μm from the cell. Ca2+-free solution had no added CaCl2 and was supplemented with 1 mM EGTA. Intracellular Ca2+ stores were depleted by 15-min pretreatment in Ca2+-free solution. Control of the Ca2+ environment was verified in two ways. First, by showing that in a Ca2+-containing bath, application of KCI (or nicotine) in Ca2+-free pipette solution failed to produce exocytosis (Fig. 1a) or elevate [Ca2+]i, (not shown) while mobilization of internal Ca2+ by caffeine under the same conditions gave positive responses (Fig. 1a). Second, in Ca2+-depleted cells, caffeine (applied with Ca2+ in the pipette) failed to stimulate the cells, while KCl depolarization continued to give positive responses (Fig. 1a). In most experiments each cell was used as its own control.

Measurement of [Ca2+]i—[Ca2+]i was measured by ratiometric determination of indo-1 fluorescence as described previously (27–29) using an ACAS 570, confocal laser photometer (Meridian Instruments, Lansing, MI). Experimental protocols for [Ca2+]i determination paralleled those used during monitoring of exocytosis.

Measurement of Cellular CAMP Content—For determination of CAMP content, chromaffin cells were plated at a concentration of about 5 × 103 cells (250,000 cells/dish) and used after 3 days. Culture medium was removed by washing with Krebs buffer solution, and 100 nM PACAP in Krebs was added to the dish for various time periods from 0 to 30 s. The reaction was stopped and cells extracted in 1 ml of ice-cold trichloroacetic acid (6%). The extract was centrifuged (3000 × g, 15 min) and the pellet used for protein determination as described (30). The supernatant was extracted with ethyl ether, evaporated to dryness, and the residue used for CAMP determination using a DuPont NEN radioimmuno assay kit according to the protocols supplied by the manufacturer.

RESULTS

PACAP Requires Extracellular Ca2+ for Exocytosis—Fig. 2a shows a representative amperometric recording of exocytotic events stimulated in an individual rat chromaffin cell by a 10-s application of 100 nM PACAP from an ejection pipette aimed at the cell. Both the bath and pipette solutions contained 2.5 mM Ca2+. Exocytosis began 6.8 ± 0.96 s (25 cells) after the start of PACAP application and continued for a minute or more after agonist application. This pattern of stimulation is much different than exocytosis produced by depolarization with KCl (Refs. 16 and 31 and see Fig. 1a) or stimulation of nicotinic receptors (below), both of which cause an almost immediate and brief (20–30 s) burst of exocytotic events.

The role of Ca2+ entry from the external medium in PACAP-induced exocytosis was examined by applying PACAP in a nominally Ca2+-free pipette solution to chromaffin cells in a 2.5 mM Ca2+-containing bath solution. PACAP (100 nM for 30 s) produced no exocytosis during the application period (Fig. 2b). However, immediately after the cessation of peptide application, there was a massive exocytotic response as the Ca2+-free pipette solution was replaced by the Ca2+-containing bath solution. Exocytosis continued for several minutes (not shown).

Exocytosis by PACAP in Cells Depleted of Internal Ca2+ Stores—To confirm the utilization of external Ca2+ in PACAP-evoked exocytosis experiments were performed with cells depleted of internal Ca2+ by maintaining cells in Ca2+-free (1 mM EGTA) bath solution for 15 min prior to and during PACAP application. Ten second application of PACAP plus Ca2+ to Ca2+-depleted cells caused a burst of exocytosis with typical latency (Fig. 2c). The duration of PACAP-evoked exocytosis in Ca2+-depleted cells was much less compared with cells with normal Ca2+ stores (compare Fig. 2, a and c).

PACAP-evoked Elevation of [Ca2+]i—The effects of PACAP on [Ca2+]i were determined in parallel with exocytosis experiments using identical protocols. Fig. 3a shows that a 10-s application of PACAP to an indo-1-loaded chromaffin cell caused an increase in [Ca2+]i that exhibits a latency to onset and prolonged duration consistent with PACAP stimulated exocytosis. In some chromaffin cells, the initial rise in [Ca2+]i was followed by fluctuating [Ca2+]i, after a brief application of PACAP (see Fig. 4 for example). In six cells monitored for 1 min or longer after PACAP application, the average latency to the beginning of [Ca2+]i elevation was 7.9 ± 0.8 s. The mean peak was 398 ± 35 nm and in four of the six cells [Ca2+]i remained elevated for the duration of recording (352 ± 29 nm at 60 s). The remaining two cells exhibited Ca2+ fluctuations throughout the recording period.

When [Ca2+]i was monitored after application of PACAP in a Ca2+-free pipette solution (Fig. 3b), there was no change in [Ca2+]i until after cessation of peptide application, identical to the pattern of exocytosis produced under the same conditions. Finally, the effects of PACAP on [Ca2+]i were determined in cells depleted of internal Ca2+ stores (Fig. 3c). The rapid decline of [Ca2+]i, under these conditions is consistent with a more rapid sequestration of Ca2+ into depleted internal stores and confirms the role of external Ca2+ entry in PACAP-evoked catecholamine secretion.

Effects of Ca2+ Channel Antagonists on PACAP Action—We used a pharmacological approach to discriminate between the delayed but long lasting responses stimulated by PACAP and the immediate brief effects of depolarizing stimuli. Among sev-
sustained elevation of [Ca\textsuperscript{2+}] caused a fluctuating rather than the sustained elevation seen with PACAP. In this example, PACAP stimulation of exocytosis requires external Ca\textsuperscript{2+}. Exocytotic events recorded from chromaffin cells in 2.5 mM Ca\textsuperscript{2+}-containing bath solution were stimulated by a 10-s application of 100 nM PACAP plus Ca\textsuperscript{2+} (a) or by 30-s application of PACAP in Ca\textsuperscript{2+}-free pipette solution (b). In (c) the records were obtained in Ca\textsuperscript{2+}-free (1 mM EGTA) bath solution after a 15-min pretreatment to deplete internal Ca\textsuperscript{2+} stores. Horizontal bars indicate the period of PACAP application. The traces in (a) and (b) are representative of 22 experiments and those in (c) from 7 observations.

FIG. 2. PACAP stimulation of exocytosis requires external Ca\textsuperscript{2+}. Exocytotic events recorded from chromaffin cells in 2.5 mM Ca\textsuperscript{2+}-containing bath solution were stimulated by a 10-s application of 100 nM PACAP plus Ca\textsuperscript{2+} (a) or by 30-s application of PACAP in Ca\textsuperscript{2+}-free pipette solution (b). In (c) the records were obtained in Ca\textsuperscript{2+}-free (1 mM EGTA) bath solution after a 15-min pretreatment to deplete internal Ca\textsuperscript{2+} stores. Horizontal bars indicate the period of PACAP application. Traces are representative of six to eight observations under each condition.

Because PACAP is established as a potent stimulator of adenylyl cyclase and cAMP formation, we questioned whether forskolin, which also stimulates adenylyl cyclase, would have secretory actions similar to PACAP. Application of forskolin (10 \mu M for 10 s) produced a long-lasting secretory response in 2.5 mM Ca\textsuperscript{2+}-medium (Fig. 5a). The delay between beginning of forskolin application and detection of the first exocytotic event was 7.5 ± 0.95 s (28 cells). When forskolin was administered in a nominally Ca\textsuperscript{2+}-free pipette solution, exocytotic events were detected only after cessation of the Ca\textsuperscript{2+}-free solution (Fig. 5b). Application of forskolin plus Ca\textsuperscript{2+} to Ca\textsuperscript{2+}-depleted chromaffin cells, like PACAP, stimulated exocytosis only during the period when Ca\textsuperscript{2+} was available to the cells (Fig. 5c).

When tested on indo-1-loaded chromaffin cells, forskolin caused a rise in [Ca\textsuperscript{2+}], that was similar to that produced by PACAP. The average peak [Ca\textsuperscript{2+}], was 322 ± 29 nM with a latency of 6.7 ± 1 s (n = 5). However, forskolin typically produced fluctuations in [Ca\textsuperscript{2+}], (see Fig. 6b for example) rather than the sustained elevation seen with PACAP. In this regard, it was noted that forskolin-evoked exocytosis was qualitatively similar to PACAP in terms of latency, use of external Ca\textsuperscript{2+}, and sensitivity to Zn\textsuperscript{2+} (Fig. 6a). However, compared with PACAP, forskolin-evoked exocytosis was less robust with fewer total exocytotic events (compare Fig. 6a and Fig. 4a). These data suggest that PACAP-evoked catecholamine secretion has a prominent, but not exclusive, cAMP-mediated mechanism.

Characteristic Delay in Onset of Exocytosis by PACAP—In all
tests performed, the latency between beginning of agonist application and detection of the first exocytic event appeared to be most closely correlated to the mechanism of agonist action (Table I). Nicotine and excess KCl, which act by depolarization and opening of voltage-dependent Ca\(^{2+}\) channels, cause secretion to occur almost instantaneously. The observed delay of a few hundred ms with these agonists reflects the positioning of the ejection pipette 20 \(\mu\)m from the cells and the resulting delay in changing the environment around the cell. Under these same conditions, agonists which mobilize internal Ca\(^{2+}\), muscarine, and caffeine, exhibited a latency between 2 and 3 s, while PACAP (or forskolin) acting presumably through a

cAMP-dependent phosphorylation cascade, required approximately 7 s to evoke secretion.

To determine if the time required for cAMP elevation could account for the latency of PACAP effects, we monitored cAMP levels during PACAP exposure. PACAP required 5 s to significantly increase cAMP levels compared with unstimulated controls (Fig. 7). The small and variable increase in cAMP observed at 2.5-s exposure to PACAP was not statistically significant. cAMP levels appeared to reach a plateau at 5 s and remained at about the same level during PACAP exposure for



**DISCUSSION**

Several lines of evidence have been presented to define the secretory mechanism of PACAP, a non-cholinergic co-transmitter in the adrenal medulla. The secretory mechanism of PACAP appears distinctly different from acetylcholine acting through nicotinic and muscarinic receptor stimulation. PACAP-evoked exocytosis and elevated \([Ca^{2+}]\) occurred only after a pronounced latency and required the presence of extracellular \(Ca^{2+}\). The time course of PACAP-stimulated CAMP elevation and the close correspondence between effects of PACAP and forskolin support the conclusion that PACAP actions are mediated by \(Ca^{2+}\)-dependent activation of protein kinases.

PACAP (or forskolin) stimulated exocytosis and elevated \([Ca^{2+}]\), only when external \(Ca^{2+}\) was present, indicating that the peptide causes \(Ca^{2+}\) entry into chromaffin cells. The absence of exocytosis and elevated \([Ca^{2+}]\) during 30-s application of PACAP without \(CaCl_2\) also shows that \(Ca^{2+}\) influx is required for PACAP-evoked catecholamine secretion. The pronounced increase in \([Ca^{2+}]\), and exocytosis upon cessation of intracellular second messengers during the application period, coupled with high affinity binding of PACAP and slow dissociation from its receptors, producing an exaggerated response as the pipette solution was displaced by the \(Ca^{2+}\) containing bath solution. The mechanism of \(Ca^{2+}\) entry following PACAP application was different than that produced by acetylcholine. Acetylcholine effects were sensitive to block of L-type \(Ca^{2+}\) channels by nifedipine, but PACAP effects were not. This is somewhat different from PACAP effects in mass cultures of porcine chromaffin cells, which were reported to be inhibited by nifedipine (14). Neither agonist was opposed by block of N-type channels by \(\omega\)-conotoxin. The finding that 100 \(\mu M\) \(Zn^{2+}\) discriminates between PACAP and other secretagogues that stimulate \(Ca^{2+}\) entry supports the idea that PACAP promotes \(Ca^{2+}\) entry by a distinct mechanism. One possibility is that PACAP causes a phosphorylation-dependent recruitment of channels not active during nicotinic or KCl induced depolarization. While forskolin-evoked exocytosis was qualitatively similar to that produced by PACAP, forskolin consistently produced fewer exocytotic events. This may be due to the reported ability of PACAP to stimulate the inositol lipid cascade along with elevated CAMP levels (33). Multiple signaling pathways stimulated by PACAP but not forskolin would account for the more robust secretion by PACAP.

The most intriguing characteristic of PACAP stimulation was the approximate 7-s delay between application and onset of action. Only a fraction of the latency could be attributed to time required for the pipette solution to displace bath solution around an individual cell. Delay intrinsic to the protocol was less than 0.5 s as indicated by the latency when depolarizing stimulus was administered. Receptor-mediated signal transduction is unlikely to account for much of the latency, since nicotinic receptor activation produced an almost immediate response. The observed latencies appear to be closely related to the intracellular mechanism involved in stimulating catecholamine secretion. Agents that mobilize internal \(Ca^{2+}\) (muscarine and caffeine) acted within 2–3 s, while agents which elevate CAMP (forskolin and PACAP) required about 7 s to produce exocytosis. Receptor-mediated production of second messengers is not likely to account for the latency for two reasons. First, both muscarine and PACAP stimulate complex second messenger pathways, but produced exocytosis with significantly different latencies. Second, caffeine, which mobilizes internal \(Ca^{2+}\) but does not act through a plasma membrane receptor, acts with the same delay as muscarine, while forskolin, which elevates CAMP independent of membrane receptors, acts with the same delay as PACAP. These observations, cou-

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**TABLE I**

| Secretagogue | Proposed mechanism | Number of cells | Latency to onset (s) |
|--------------|--------------------|----------------|---------------------|
| 10 \(\mu M\) nicotine | Depolarization | 14 | 0.55 ± 0.09 |
| 35 \(mm\) KCl | Internal \(Ca^{2+}\) release | 22 | 0.36 ± 0.06* |
| 30 \(\mu M\) muscarine | CAMP production | 15 | 2.2 ± 0.25* |
| 10 \(mM\) caffeine | Depolarization | 12 | 2.9 ± 0.38* |
| 100 \(nm\) PACAP |  | 25 | 6.8 ± 0.96* |
| 10 \(\mu M\) forskolin |  | 28 | 7.5 ± 0.95* |

* No difference compared to other secretagogue proposed to act by the same mechanism, \(p > 0.1\), Student’s \(t\) test.
*\(^b\) \(p < 0.001\) compared with nicotine.
*\(^c\) \(p < 0.001\) compared with muscarine.

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**FIG. 8. Inhibition of protein kinase A blocks PACAP-evoked exocytosis.** Chromaffin cells were stimulated by 10-s application of PACAP (100 nm) or 500-ms application of nicotine (10 \(\mu M\)) as indicated from an ejection pipette. Exocytosis was detected amperometrically in control (solid bars) and 300 \(\mu M\) \(R_{-}\)-cAMPS following 30-min pretreatment (shaded bars). The counting period was 60 s for PACAP- and 30 s for nicotine-stimulated responses. Note that nicotine-evoked responses were complete within 30 s, while PACAP-evoked responses typically continued beyond the recording period. Bars represent the mean (±S.E.) of three experiments. *, \(p < 0.05\), unpaired Student’s \(t\) test. n.s., not significant.

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**FIG. 7. PACAP-stimulated elevation of CAMP.** Chromaffin cells were exposed to 100 \(nm\) PACAP for the time indicated and CAMP content determined. Zero time represents untreated controls. Symbols represent the mean (±S.E.) of three to four observations at each time point. *, \(p < 0.05\) compared with control, unpaired Student’s \(t\) test.
The approximate 5-s latency for PACAP-evoked exocytosis support the idea that PACAP production and activation of protein kinase A are involved in stimulating Ca\(^{2+}\) entry and exocytosis. However, the mechanism of this action remains unknown.

In conclusion, the results presented here demonstrate the importance of multiple transmitters acting through cholinergic and non-cholinergic pathways in the adrenal synapse. PACAP stimulation of catecholamine secretion is not redundant or simply modulatory, but occurs independent of other secretagogues via mechanisms distinct from cholinergic receptor coupled pathways. Thus, peptide transmission is likely to maintain catecholamine secretion during periods of stress or situations where cholinergic receptors desensitize or cholinergic transmission otherwise fails.

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