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Research Article

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Peptidergic Activation of Transcription and Secretion in Chromaffin Cells

cis and *trans* Signaling Determinants of Pituitary Adenylyl Cyclase-activating Polypeptide (PACAP)

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Abstract

Pituitary adenylyl cyclase-activating polypeptide (PACAP) is a potent endogenous secretagogue for chromaffin cells. Chromogranin A is the major soluble core component in secretory vesicles. Since chromogranin A is secreted along with catecholamines, we asked whether PACAP regulates expression of the chromogranin A gene in PC12 rat chromaffin cells, so as to resynthesize the just-secreted protein, and whether such biosynthetic regulation is coupled mechanistically to catecholamine secretion. PACAP activated the endogenous chromogranin A gene by four- to fivefold. Proportional results (seven- to eightfold activation) were obtained with a transfected 1,200-bp mouse chromogranin A promoter/luciferase reporter construct. A series of chromogranin A promoter 5' deletion mutant/luciferase reporter constructs narrowed down the PACAP response element to a proximal region containing the cAMP response element (CRE box), at (−71 bp)5'-TGACGTAA-3' (−64 bp). Site-directed point mutations of the CRE site suppressed PACAP-induced *trans*-activation of the promoter. Thus, the proximal CRE box is entirely necessary for the chromogranin A promoter response to PACAP. Transfer of the CRE box to a neutral, heterologous promoter also conferred activation by PACAP, suggesting that the CRE domain is also sufficient to mediate the transcriptional response to PACAP. Expression of a dominant-negative mutant (KCREB) of the CRE-binding factor CREB markedly diminished *trans*-activation of the chromogranin A promoter by PACAP. Cotransfection of expression plasmids encoding the protein kinase A inhibitor, or an inactive protein kinase A (PKA) catalytic β subunit, inhibited both forskolin and PACAP activation of chromogranin A transcription, revealing that PACAP-induced *trans*-activation is highly dependent on PKA. By contrast, inhibition of protein kinase C (by chronic exposure to phorbol ester) had no effect on transcriptional activation by PACAP. The potent PACAP/vasoactive intestinal peptide (VIP) type I receptor antagonist PACAP6-38 impaired both chromogranin A transcription

or catecholamine secretion triggered by PACAP38, while the PACAP/VIP type II receptor antagonist (p-Chloro-D-Phe⁶, Leu¹⁷)-VIP had little or no ability to antagonize the PACAP38 effect. The agonist VIP was ~ 100- to 1,000-fold less potent than PACAP in stimulating either secretion or transcription. Thus, PACAP-evoked chromogranin A transcription and catecholamine secretion are likely mediated by the PACAP/VIP type I receptor isoform. Although the calcium channel antagonists Zn²⁺ (100 μ M), nifedipine (10 μ M), or ruthenium red (10 μ M), or the cytosolic calcium chelator BAPTA-AM (50 μ M) each strongly impaired PACAP-induced secretion, transcriptional activation of chromogranin A remained unaltered. Therefore, we propose that PACAP signals to chromogranin A transcription through the CRE in *cis*, and through PKA and CREB in *trans*. By contrast, a pathway involving cytosolic calcium entry through L-type voltage-dependent channels is required for PACAP to evoke catecholamine secretion. (*J. Clin. Invest.* 1998, 101:863–876.) Key words: PC12 • chromaffin • PACAP • catecholamine • pheochromocytoma

Introduction

The biologically active peptide pituitary adenylyl cyclase-activating polypeptide (PACAP),¹ the latest discovered member of the secretin/glucagon/vasoactive intestinal peptide (VIP) regulatory peptide family, was originally identified in hypothalamus as a stimulator of adenylyl cyclase in the anterior pituitary (1, 2). This neuropeptide occurs in both 27- (PACAP27) and 38- (PACAP38) amino acid forms, and bears partial sequence homology to VIP. So far, three distinct PACAP/VIP receptor (PVR) subtypes have been identified and cloned (3-6). The PACAP type I receptor (PVR1) is selective for PACAP, but also recognizes VIP at low affinity. PACAP type II receptors have equal affinity for PACAP and VIP, and have been divided into PVR2 and PVR3 subtypes. PVR1 is coupled to activation of both adenylyl cyclase and phospholipase C, likely through guanine-nucleotide-binding protein Gs and Gq isoforms (7). In contrast, PVR2 and PVR3 are apparently linked only to activation of adenylyl cyclase. However, these receptors may also couple to additional effectors. For example, a PACAP-induced synaptic current in *Drosophila* is mediated by co-activation of the low molecular weight G protein kinase Ras and cAMP signaling pathways

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1. Abbreviations used in this paper: CAT, chloramphenicol acetyltransferase; CRE, cAMP response element; PACAP, pituitary adenylyl cyclase-activating polypeptide; PKA, inactive protein kinase A; PKI, protein kinase A inhibitor; PVR, PACAP/VIP receptor; PVR1, PACAP type I receptor; TK, thymidine kinase; VIP, vasoactive intestinal peptide.

(8). PACAP seems to regulate hormone production and secretion in several endocrine organs, including the pituitary, thyroid, and the pancreas (7, 9–11).

In secretory granules of chromaffin cells or postganglionic sympathetic axons, catecholamines are costored and cosecreted by exocytosis along with several soluble proteins and peptides, including the chromogranin/secretogranin family. These acidic proteins are found in secretory vesicle cores of virtually all neuroendocrine and neuronal cells (12–14). The most widely expressed member of this protein family is the 48-kD chromogranin A, to which both intracellular and extracellular functions have been ascribed. Intracellular functions include binding of calcium, ATP and catecholamines within the hormone storage vesicle core (15–17), inhibition of prohormone processing enzymes (18), and a trafficking role in guiding other secretory proteins into dense core vesicles within the regulated secretory pathway (19, 20). Extracellular functions include generation of several bioactive peptides by proteolytic processing (21–26) such as the vascular smooth muscle relaxing factor vasostatin (25) or the catecholamine release-inhibitor catestatin (26).

In addition to the classic preganglionic neurotransmitter acetylcholine, which is released during splanchnic nerve activity, noncholinergic transmitters such as VIP contribute to the secretory and biosynthetic responses of adrenal medullary chromaffin cells (27–31). More recently, PACAP was described as substantially more potent than VIP in stimulating both catecholamine secretion and tyrosine hydroxylase (TH) gene expression in chromaffin cells of several species (32–38). A dense network of PACAP-immunoreactive fibers innervates the adrenal gland, and abundant PACAP receptors occur on the surface of chromaffin cells (39, 40). Chromaffin cell roles for PACAP distinct from the functions of acetylcholine have been suggested (36, 38). In contrast to nicotine, whose secretory response shows rapid desensitization (41, 42), PACAP can evoke a long-lasting catecholamine secretion, for up to several hours (36). Taken together, these findings support the idea that PACAP is an important noncholinergic neurotransmitter (or cotransmitter) modulating catecholamine synthesis and the secretory response of chromaffin cells. However, mechanisms whereby PACAP might influence the biosynthesis of the just-secreted proteins and neuropeptides of the secretory granule have not yet been elucidated.

In this study, we questioned whether PACAP regulates expression of the chromogranin A gene in PC12 rat chromaffin cells, so as to resynthesize the just-secreted protein, and whether this gene regulation is coupled mechanistically to catecholamine secretion. Our results suggest that PACAP signals to both chromogranin A transcription and catecholamine secretion through the PVR1 receptor. The chromogranin A gene is activated by PACAP predominantly through the proximal cAMP response element (CRE) in *cis*, and through protein kinase A and the transcription factor CREB in *trans*. PACAP requires extracellular Ca^{2+} entry through L-type voltage-gated channels to stimulate catecholamine secretion. By contrast, PACAP-induced transcriptional activation of the chromogranin A gene does not seem to depend on increments of intracellular calcium concentration. Taken together, our results indicate that PACAP uses divergent signaling pathways in evoking catecholamine secretion versus chromogranin A transcription in PC12 cells. Moreover, this work supports the hypothesis that PACAP is a potent noncholinergic neurotransmitter con-

trolling both secretory and biosynthetic activities of sympathoadrenal cells.

Methods

Cell culture. Passage 8 PC12 rat pheochromocytoma cells (43) obtained from David Schubert (Salk Institute, La Jolla, CA), were cultured in high-glucose Dulbecco's modified Eagle's medium (DME) supplemented with 10% heat-inactivated horse serum and 5% heat-inactivated fetal bovine serum (FBS; Gemini Bioproducts, Calabasas, CA), streptomycin (100 μ g/ml), and penicillin (100 U/ml).

Catecholamine secretion. Catecholamine secretion from PC12 cells was performed as previously described (44). Briefly, cells were grown on poly-D-lysine (Sigma Chemical Co., St. Louis, MO)-coated six-well culture dishes, loaded for 2 h with 1 μ Ci [3 H]-L-norepinephrine (DuPont-NEN, Boston, MA), and washed with saline buffer (150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10 mM Hepes, pH 7.4). Cells were subsequently stimulated for 15 min with the indicated concentrations of secretagogue in saline buffer. [3 H]-L-norepinephrine secretion was calculated as a percent of total radioactivity (present in the cells before stimulation), where total radioactivity is the sum of the amount released plus the amount remaining in the cells.

Chromogranin A promoter-reporter constructs and expression plasmids. Promoter fragment positions are numbered relative to the major transcriptional (cap) site as +1. For example, pXP1200 contains 1,200 bp of the mouse chromogranin A promoter (5' flanking region) fused to a luciferase (Luc) reporter in the promoterless luciferase reporter vector pXP1. Construction of mouse chromogranin A promoter/luciferase reporter plasmids pXP1200, pXP821, pXP682, pXP529, pXP339, pXP258, pXP181, pXP77, pXP61, and pXP43 was described previously (45). Site-directed mutants of chromogranin A promoter expression (plasmid pXP100, mutations M9 to M16) were generated as described previously (46). To study the role of the CRE box in isolation, the mouse chromogranin A promoter's CRE domain (TGACGTA), or a consensus CRE (TGACGTCA), were ligated upstream of the heterologous TK (Herpes simplex virus thymidine kinase) promoter in pTK-LUC, as described previously (45, 47). pRSV-KCREB (gift of Richard H. Goodman, Oregon Health Science University, Portland, OR) expresses a dominant negative (DNA binding domain) point mutant of CREB, and was described previously (48). pRSV-protein kinase A inhibitor (PKI) is the expression vector for the heat stable inhibitor PKI of cAMP-dependent protein kinase A; pRSV- Δ PKI expresses the inactive PKI mutant of the protein kinase A inhibitor PKI (49). Plasmid pRSV- Δ PKA β expresses a mutant (inactive) β catalytic subunit of protein kinase A (50). These plasmids were kindly supplied by Richard A. Maurer (University of Iowa, Iowa City, IA).

Transient transfection/cotransfection studies. Supercoiled plasmid DNA for transfection was grown in *Escherichia coli* strain DH-5, and purified on columns (QIAGEN Inc., Chatsworth, CA). 24 h before transfection, PC12 cells were split onto poly-D-lysine-coated 6-well Falcon plates, at 40–50% confluence. Cells were transfected with 2–6 μ g of supercoiled plasmid DNA per well, using the lipofection method (Lipofectamine or Lipofectin, 5 μ g/ml; Life Technologies Inc., Gaithersburg, MD) or the polycation method (Superfect; QIAGEN Inc.). When the reporter or *trans*-activator plasmid did not total the requisite amount of DNA, the balance was composed of supercoiled pBluescript (Stratagene, Inc., La Jolla, CA). To correct for transfection efficiency differences between plasmids, some transfections also involved cotransfection with a control reporter plasmid, pRSV-chloramphenicol acetyltransferase (CAT). This plasmid expresses the CAT reporter driven by the Rous Sarcoma Virus promoter (51). 24 h after transfection, PC12 were then treated for an additional 18–20 h (unless otherwise described) with the indicated concentrations of agonists. Cells were harvested as previously described (52) and assayed for luciferase reporter activity (in relative light units [RLU]) (53); CAT reporter activity (dpm [14 C]-acetylated chloramphenicol formed; 51), or protein concentration (54). Lu-

ciferase results were normalized to either cell CAT or cell protein content.

RNA extraction and Northern blots. Total RNA was isolated from PC12 cells using the guanidinium thiocyanate extraction method (RNAzolB; TEL-TEST, Inc., Friendswood, TX), and quantified by UV absorption (A260) and A260/A280 absorbance ratio. 15 µg of total cellular RNA were size fractionated on denaturing 1% formaldehyde-agarose gels and transferred to a nitrocellulose membrane. The integrity of the RNA was judged by the appearance of 28S and 18S rRNA bands on the ethidium bromide-stained gel. Northern blots were done essentially as described previously (46). Random primer-labeled cDNA probes (Life Technologies Inc.) were a 1.6-kbp rat chromogranin A cDNA (55) and a 381-bp mouse cyclophilin cDNA (56) used as a normalizing probe for a constitutively expressed mRNA. Between probes, membranes were stripped by boiling for 2–3 min in 0.1 × SSC, 0.1% SDS, before rehybridization.

Reagents. Ryanodine, forskolin, phorbol 12-myristate 13-acetate (PMA) and BAPTA-AM were obtained from Calbiochem Corp. (La Jolla, CA). Nicotine, nifedipine, dibutyryl-cAMP, ruthenium red, and ZnCl₂ were from Sigma Chemical Co. (St. Louis, MO). α-[³²P]-dCTP was from DuPont-NEN.

Peptides. Synthetic PACAP-38 and VIP were obtained from Peninsula Laboratories, Inc. (Belmont, CA). Synthetic PACAP6-38 and [p-Chloro-D-Phe⁶, Leu¹⁷]-VIP were from Bachem California (Torrance, CA). For some experiments, the PACAP38 used was synthesized on an NPS 4000 semi-automated multichannel peptide synthesizer by the solid phase synthetic approach using f-moc protection chemistry (Core Peptide Facility, University of California at San Diego, La Jolla, CA). PACAP38 was then purified by reverse-phase HPLC on a semi-preparative Supelcosil LC-18 column (5 µm, 10 × 250 mm; Supelco Inc., Bellefonte, PA), monitoring at A214 nm. Further purification was achieved by a second cycle of reverse-phase HPLC on a Supelcosil LC-318 semi-analytical column (5 µm, 4.6 × 250 mm). Authenticity and purity (> 98%) of PACAP38 were verified by rechromatography (as just described), as well as electrospray ionization and MALDI mass spectrometry (Scripps Research Institute Mass Spectrometry, La Jolla, CA).

Statistics. Values are given as the mean ± SEM of triplicate determinations. Statistical significance was assessed by Student's *t* test using Statworks for Macintosh (Cricket Software, Malvern, PA). Differences were considered significant when *P* < 0.05.

Results

Induction of chromogranin A mRNA in response to PACAP. Steady state chromogranin A mRNA levels indicated that

PACAP38 stimulates transcription of the endogenous chromogranin A gene, in a time-dependent manner (Fig. 1). Chromogranin A mRNA expression was significantly increased by 3 h (1.62-fold over basal) of PACAP38 exposure, and reached a maximum of 4.87-fold stimulation at 48 h.

PACAP triggers both catecholamine secretion and trans-activation of a transfected chromogranin A promoter. PACAP-38 evoked catecholamine secretion from PC12 cells in concentration-dependent manner (Fig. 2A). Increased catecholamine release became significant at > 1 nM peptide and reached a maximum at 0.5–1 µM peptide, with EC₅₀ ~ 12 nM. In contrast, the activity of a transfected 1,200-bp chromogranin A promoter/reporter plasmid (pXP1200) was increased significantly at 0.1 nM PACAP38, but a maximal response was only achieved at > 5 µM peptide (Fig. 2B). In 29 separate experiments, the induction of the transfected chromogranin A promoter by PACAP (250 nM) was 5.5 ± 0.64-fold.

As shown in Fig. 2C, PACAP-induced *trans*-activation of the chromogranin A promoter was time dependent. Promoter activity was already increased after 3 h (5.44-fold stimulation over basal) and reached a peak value between 6 and 24 h of treatment (approximately seven- to eightfold stimulation over basal). Promoter activity then declined after 24–48 h of treatment, to a value of 2.17-fold over basal.

Localization of PACAP-responsive elements within the chromogranin A promoter. The PACAP response was tested on a series of transfected chromogranin A promoter 5' deletion/luciferase reporter constructs (Fig. 3A). Deletion of two promoter regions, from –1,200 to –821 bp and from –77 to –61 bp upstream of the cap site, caused substantial decrements in the PACAP38 response: by 43% for the distal domain, and by 77% for the proximal domain. By contrast, deletion of the region from –181 to –77 bp increased the PACAP38 effect (to 3.32-fold of basal). This experiment reveals the crucial role of a small proximal promoter domain (from –77 to –61 bp) in the PACAP38 response; this domain contains the chromogranin A promoter's functional cAMP response element: [–71 bp]5'-TGACGTAA-3' [–64 bp] (46).

Dense scanning site-directed mutagenesis of the CRE domain within the chromogranin A promoter. Site-directed point mutations of the chromogranin A CRE site (TGACGTAA), within the mouse chromogranin A 100-bp promoter/luciferase reporter construct, were tested for their effect on PACAP38-induced transcriptional activation.

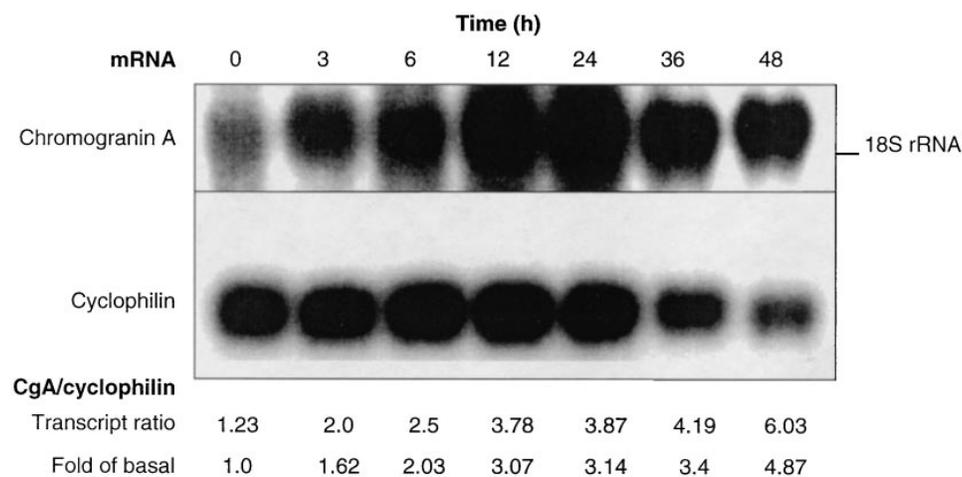


Figure 1. Northern blot analysis of the time-dependent effect of PACAP on chromogranin A mRNA. PC12 cells were treated with 250 nM PACAP38 for the indicated times. Cells were harvested and chromogranin A mRNA levels were analyzed by Northern blot. The cyclophilin probe recognizes a constitutive or housekeeping mRNA, at the same time point. 18S rRNA, position (mobility) of the 18S form of ribosomal RNA on the ethidium bromide-stained gel. Numerical results on the lower panel represent the mean relative abundance of chromogranin A mRNA (ratio of integrated density of chromogranin A mRNA/integrated density of cyclophilin mRNA).

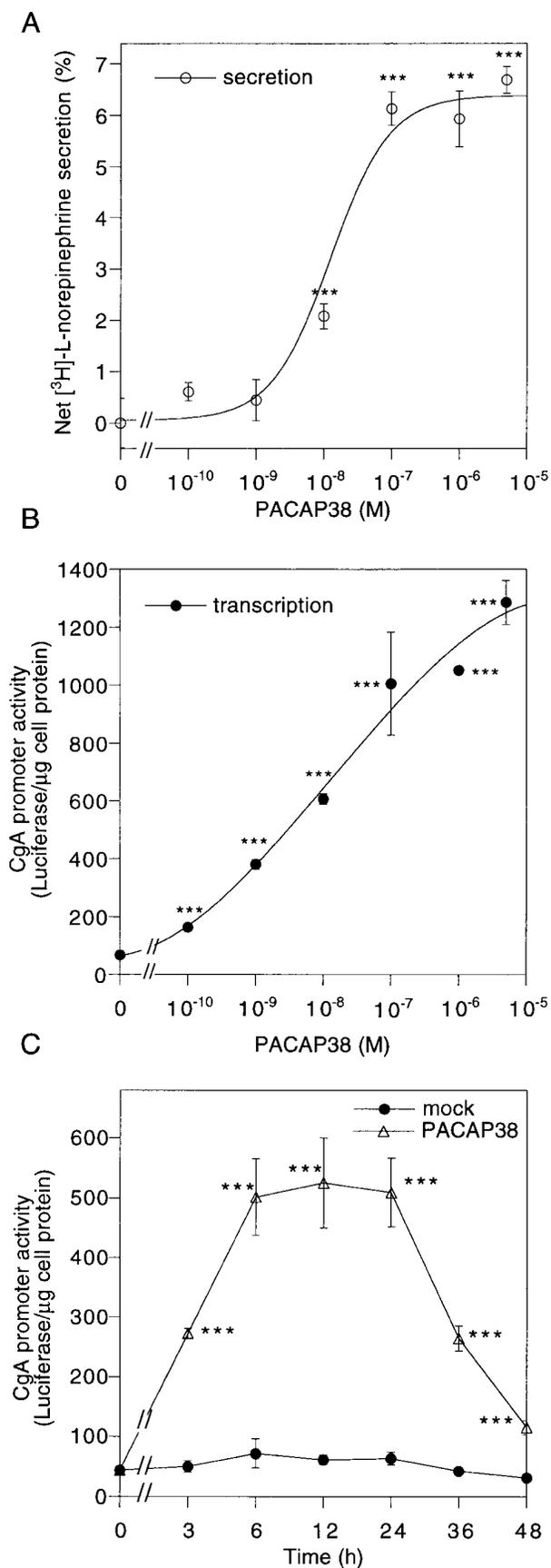


Figure 2. Stimulating effect of PACAP on catecholamine secretion and transcriptional activation of a transfected chromogranin A pro-

mo-terase reporter construct (wild-type pXP100), profoundly affected PACAP38-induced *trans*-activation of the promoter (Fig. 3 B). PACAP38-activation of transfected CRE mutant promoter/reporter constructs M9, M12, M13, M15, and M16 was reduced by 62, 69, 90, 61, or 35%, respectively versus control (wild-type pXP100). In contrast, CRE mutation M14, bearing an 8-bp consensus CRE sequence (TGACGTCA) enhanced the PACAP38 response (by 100% over wild type). Thus, mutations of the CRE away from the consensus (TGACGT[C/A]A) impaired the response to PACAP.

Stimulation of a heterologous (TK) promoter in response to PACAP, by transfer of CRE domains. To further examine the role of the CRE domain in mediating *trans*-activation by PACAP38, a single copy of a double-stranded synthetic chromogranin A CRE fragment (TGACGTAA), or a consensus CRE site (TGACGTCA), was subcloned into the enhancerless promoter/luciferase reporter plasmid pTK-Luc, just upstream (5') of the TK promoter (46). Transfection of these constructs, followed by exposure to PACAP38 (250 nM; 20 h) revealed that both the original chromogranin A CRE domain (TGACGTAA) and a consensus CRE domain (TGACGTCA) conferred the PACAP38 response onto the previously unresponsive TK promoter (Fig. 3 C). PACAP38 activation of the chromogranin A CRE (TGACGTAA)-TK construct reached a value of 44.2 ± 7.55 -fold over basal, while the consensus CRE (TGACGTCA)-TK construct was activated by 77.7 ± 6.68 -fold over basal.

*Effect of a dominant negative mutant of the CRE-binding factor CREB (mutant KCREB) on PACAP-induced *trans*-activation of the chromogranin A promoter.* Cotransfection of PC12 cells with a dominant negative (inhibitory) mutant of the CRE-binding protein CREB (mutant KCREB), together with

mo-ter. (A) Dose-dependent effect of PACAP38 on norepinephrine secretion. [³H]-L-norepinephrine-prelabeled PC12 cells were treated for 15 min with indicated concentration of PACAP38. Cells were then harvested and processed for norepinephrine secretion determination. Secretion values are expressed as percentage of net [³H]-L-norepinephrine release. Values are given as the means of triplicate determinations \pm SEM of one representative experiment. ****P* < 0.001, as compared with mock (control) treatment. (B) Dose-

dependent effect of PACAP38 on chromogranin A transcription. PC12 cells transfected with the 1,200-bp mouse chromogranin A promoter/luciferase reporter (2.5 μ g/ml), were treated for 20 h with indicated concentration of PACAP38. Cells were then harvested for luciferase activity assay and cell protein concentration. Results are expressed as luciferase reporter values normalized to microgram cell protein, representing chromogranin A promoter activity. Values are given as the means of triplicate determinations \pm SEM of one representative experiment. ****P* < 0.001, as compared with mock (control) treatment. (C) Time course of response of the transfected 1,200-bp mouse chromogranin A promoter/luciferase reporter to PACAP38. PC12 cells transfected with the 1,200-bp mouse chromogranin A promoter/luciferase reporter (2.5 μ g/ml) and incubated in culture medium in the absence (*black circles*) or the presence (*open triangles*) of PACAP38 (250 nM). Cells were then harvested for luciferase activity assay and cell protein concentration at the indicated time. Results are expressed as luciferase reporter values normalized to microgram cell protein, representing chromogranin A promoter activity. Values are given as the means of triplicate determinations \pm SEM of one representative experiment. ****P* < 0.001, as compared with mock (control) treatment.

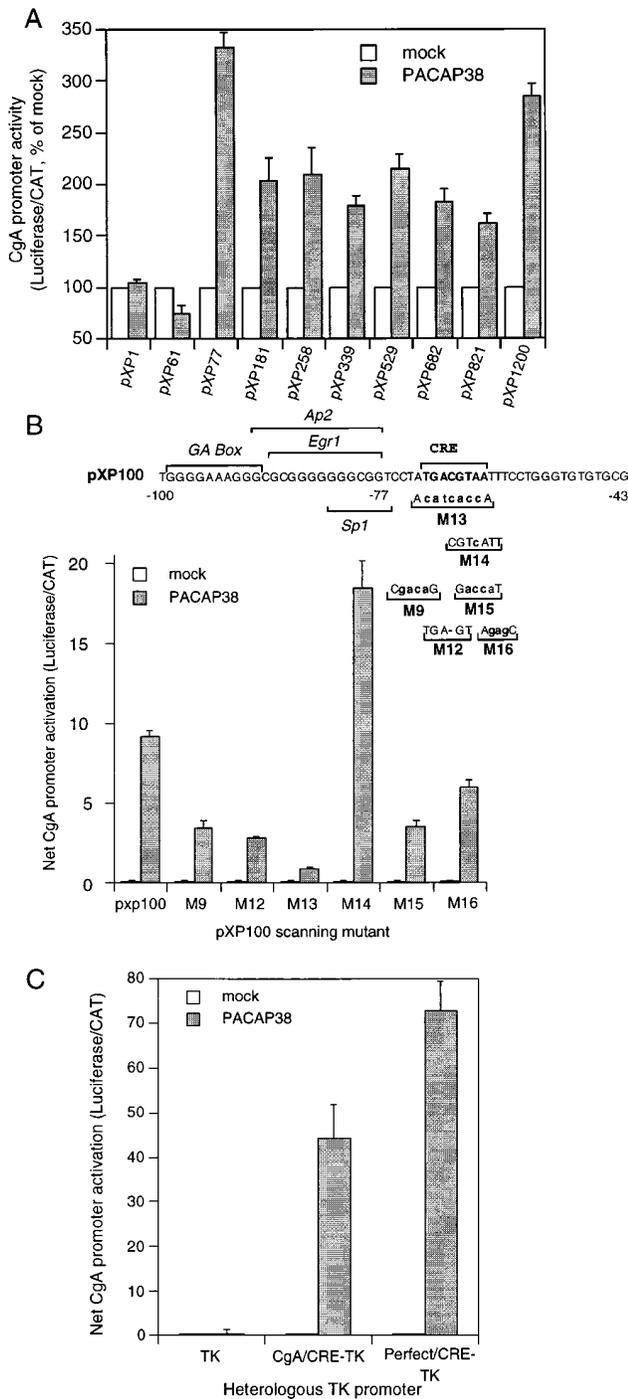


Figure 3. PACAP signals to chromogranin A transcription through the chromogranin A promoter's proximal cAMP response element (CRE domain; [-71bp]5'-TGACGTAA-3'[-64bp]). (A) Response of progressive 5' deletions of the mouse chromogranin A promoter, in promoter/luciferase constructs, to stimulation by PACAP38. Promoter deletion positions are numbered by reference to bp upstream of the mouse chromogranin A transcription initiation site. For example, pXP1200 contains 1,200 bp of the mouse chromogranin A promoter (5' flanking region) fused to a luciferase (Luc) reporter in the promoterless luciferase reporter vector pXP1. PC12 cells were transfected with promoter constructs (2.5 μ g/ml) and incubated for 20 h with or without 250 nM PACAP38. Cells were subsequently harvested and processed for luciferase and CAT activity, resulting from cotransfected pRSV-CAT (0.5 μ g/ml). Results are expressed as lu-

either a 77-bp (pXP77) or 1,200-bp (pXP1200) chromogranin A promoter/luciferase reporter, resulted in almost complete abolition of the stimulatory effects of PACAP38 (Fig. 4 A). KCREB expression also diminished the response of the isolated chromogranin A CRE (in the CRE-TK/luciferase plasmid) to cAMP by \sim 90% (Fig. 4 B), indicating the specificity of KCREB towards its target, the CRE motif.

Role of protein kinase C in transcriptional regulation of the chromogranin A promoter by PACAP. Downregulation of protein kinase C by chronic exposure to phorbol myristate acetate (1 μ M, 24 h) did not impair PACAP *trans*-activation of the transfected 1,200-bp chromogranin A promoter/luciferase reporter (Fig. 5 A). In a previous report (57), we showed that nicotinic cholinergic-stimulated transcription of chromogranin A was highly dependent upon protein kinase C. When PACAP38 (250 nM) and nicotine (500 μ M) were combined, their effects on chromogranin A transcription were approximately additive (Fig. 5 B), suggesting different signaling pathways for PACAP38 and nicotine effects on chromogranin A gene expression.

Transcriptional activation of the chromogranin A promoter by PACAP is dependent upon protein kinase A activation. Cotransfection of an expression plasmid for the protein kinase A inhibitor PKI (pRSV-PKI) with the chromogranin A 1,200-bp promoter/luciferase reporter plasmid (pXP1200) markedly reduced PACAP38- or forskolin-stimulated expression of the

ciferase/CAT activities normalized to mock (control) treatment. Values are given as the means of triplicate determinations \pm SEM of one representative experiment. (B) Effect of dense scanning site-directed mutagenesis on the response of chromogranin A promoter to PACAP38. The promoter mutant plasmids (constructs M9 to M16) or their wild-type 100-bp control (pXP100) were transfected into PC12 cells (2.5 μ g/ml). Cells were subsequently treated for 20 h with or without 250 nM PACAP38, then harvested for luciferase and CAT activities, resulting from cotransfected pRSV-CAT (0.5 μ g/ml). Results are expressed as net luciferase reporter values normalized to CAT activity representing net chromogranin A promoter activation. Values are given as the means of triplicate determinations \pm SEM of one representative experiment. pXP100 is the wild-type mouse chromogranin A promoter/luciferase reporter plasmid for these studies; in pXP100, a 100-bp mouse chromogranin A promoter is fused to a luciferase reporter. Point mutations are indicated by change of that nucleotide to lower case and bold type. In mutation M12, a point-gap (TGA-GTAA) mutation is introduced into the CRE motif (TGACG-TAA). CRE, cAMP response element; Ap2, activator protein 2 consensus motif; GA Box, purine (G/A) -rich motif; Egr1, early growth response factor motif; Sp1, stimulatory protein 1 (G/C-rich) motif. (C) Stimulation of a heterologous thymidine kinase promoter by the CRE site in response to PACAP38. PC12 cells were transfected with synthetic CRE-TK/luciferase reporter plasmids (2.5 μ g/ml) and treated for 20 h with PACAP38 (250 nM). Cells were then harvested for luciferase and CAT activity assays, resulting from cotransfected pRSV-CAT (0.5 μ g/ml). Results are expressed as net luciferase reporter values normalized to CAT activity representing net chromogranin A promoter activation. Values are given as the means of triplicate determinations \pm SEM of one representative experiment. TK, thymidine kinase (heterologous promoter); CgA/CRE-TK, the original chromogranin A CRE (TGACGTAA, that is, the CRE motif as it exists in rodent chromogranin A promoters) fused to a TK promoter driving luciferase expression; Perfect/CRE-TK, a consensus CRE motif (TGACGTCA) fused to a TK promoter driving luciferase expression.

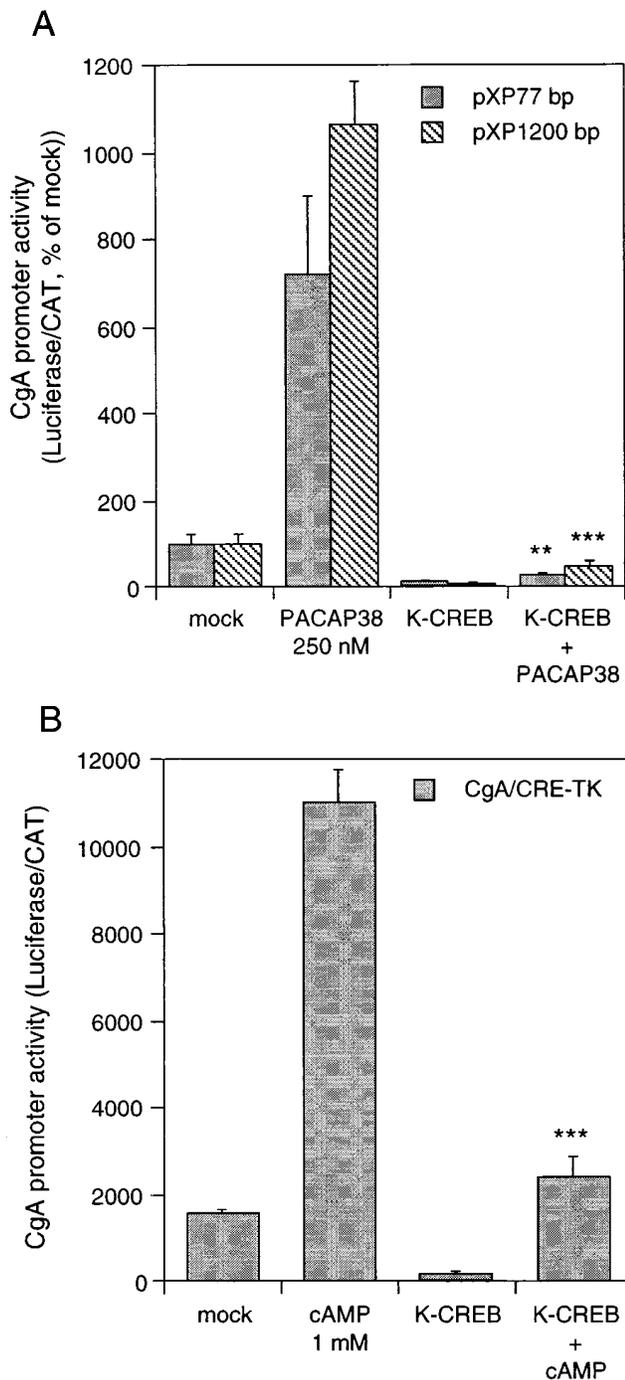


Figure 4. A dominant negative (inhibitory) mutant of the CRE-binding factor CREB (mutant KCREB) prevents PACAP-induced activation of the chromogranin A promoter, or cAMP activation of an isolated CRE. PC12 cells were cotransfected with: (A) a 77-bp mouse chromogranin A promoter (pXP77; 2 μ g/ml), or a 1,200-bp mouse chromogranin A promoter (pXP1200; 2 μ g/ml); or (B), an isolated CRE element (TGACGTAA) fused to the TK promoter, in the construct chromogranin A/CRE-TK-Luc. To achieve a constant amount of transfected DNA, cotransfections included pRSV-KCREB (2 μ g/ml) or pBluescript (2 μ g/ml). Cells were subsequently exposed to 250 nM PACAP38 (A) or 1 mM dibutyryl cAMP (B), followed by harvest for reporter assay (luciferase and CAT). Control for transfection efficiency was achieved by cotransfection of pRSV-CAT (0.5 μ g/ml). Results are expressed as luciferase reporter values normalized to CAT activity, representing chromogranin A promoter activity. Values are

chromogranin A promoter in PC12 cells (Fig. 6 A). In this experiment, stimulated chromogranin A promoter activity following PACAP38 or forskolin was reduced to 23–31% of the activity observed in the absence of PKI. In contrast, expression of the protein kinase inhibitor mutant Δ PKI (49) had little ($P > 0.05$) effect (Fig. 6 A) on the PACAP response. The mutant Δ PKI did inhibit forskolin stimulation of the chromogranin A promoter by $\sim 30\%$ (Fig. 6 A, $P = 0.033$).

Transfection of cells with an expression vector encoding an inactive β catalytic subunit of protein kinase A (pRSV- Δ PKA β) (50) impaired by $\sim 80\%$ the ability of PACAP38 to stimulate chromogranin A transcription (Fig. 6 B), indicating a dominant negative (inhibitory) effect of this Δ PKA β mutant.

Taken together, these findings suggest that protein kinase A activation is both necessary and sufficient to mediate the effect of PACAP38 on chromogranin A gene transcription.

Transcriptional activation of the chromogranin A promoter, as well as stimulation of norepinephrine secretion, are mediated essentially by the PACAP/VIP type I (PVR1) receptor isoform. To determine which PACAP/VIP receptor isoform is required for PACAP38 actions in PC12 cells, we examined the effects of: (a) PACAP6-38, a potent PVR1 competitive inhibitor (58); (b) [p-Chloro-D-Phe⁶, Leu¹⁷]-VIP, a selective PACAP/VIP type II competitive antagonist (59); or (c) VIP (which acts principally through the PACAP/VIP type II receptor), on *trans*-activation of the 1,200-bp chromogranin A promoter/luciferase reporter, as well as on catecholamine release.

The PVR1 antagonist PACAP6-38 markedly inhibited PACAP38-induced catecholamine secretion (Fig. 7 A) and also impaired chromogranin A *trans*-activation (Fig. 7 B), in a dose-dependent manner, with similar IC_{50} values (10^{-7} to 10^{-6} M). By contrast, the PACAP/VIP type II receptor antagonist [p-Chloro-D-Phe⁶, Leu¹⁷]-VIP did not significantly impair the stimulatory effects of PACAP38 on transcription (Fig. 7 B).

A modest inhibitory effect (-36% , $P < 0.05$) of [p-Chloro-D-Phe⁶, Leu¹⁷]-VIP on catecholamine secretion occurred only at a concentration of antagonist $\geq 5 \mu$ M (Fig. 7 A); this observation suggests that the PACAP/VIP type II receptor makes only a minor (if any) contribution to PACAP38 secretory effects. Indeed, at this very high concentration of competitor (e.g., $\geq 5 \mu$ M), we cannot exclude an effect of [p-Chloro-D-Phe⁶, Leu¹⁷]-VIP on the PACAP/VIP type I receptor. A low affinity of this VIP analog for the PACAP/VIP type I receptor would be consistent with the characteristic agonist affinity pattern for PVR1 (PACAP38 \approx PACAP27 \gg VIP).

The agonist VIP, which has low affinity for PVR1 but is the classic agonist of the PACAP/VIP type II receptor, was ~ 100 – $1,000$ times less potent than PACAP38 on either transcription or secretion (Fig. 7 C, see also Fig. 2, A and B), further suggesting that both secretion and transcription evoked by PACAP38 are mediated largely if not completely by the PVR1 receptor isoform. These relative potencies of PACAP versus VIP on catecholamine secretion have been observed previously (33).

Contribution of calcium to catecholamine secretion or transcriptional activation of the chromogranin A promoter by PACAP. The role of Ca^{2+} entry from the external medium in

given as the means of triplicate determinations \pm SEM of one representative experiment. *** $P < 0.001$; ** $P < 0.01$ as compared with the corresponding responses in absence of K-CREB.

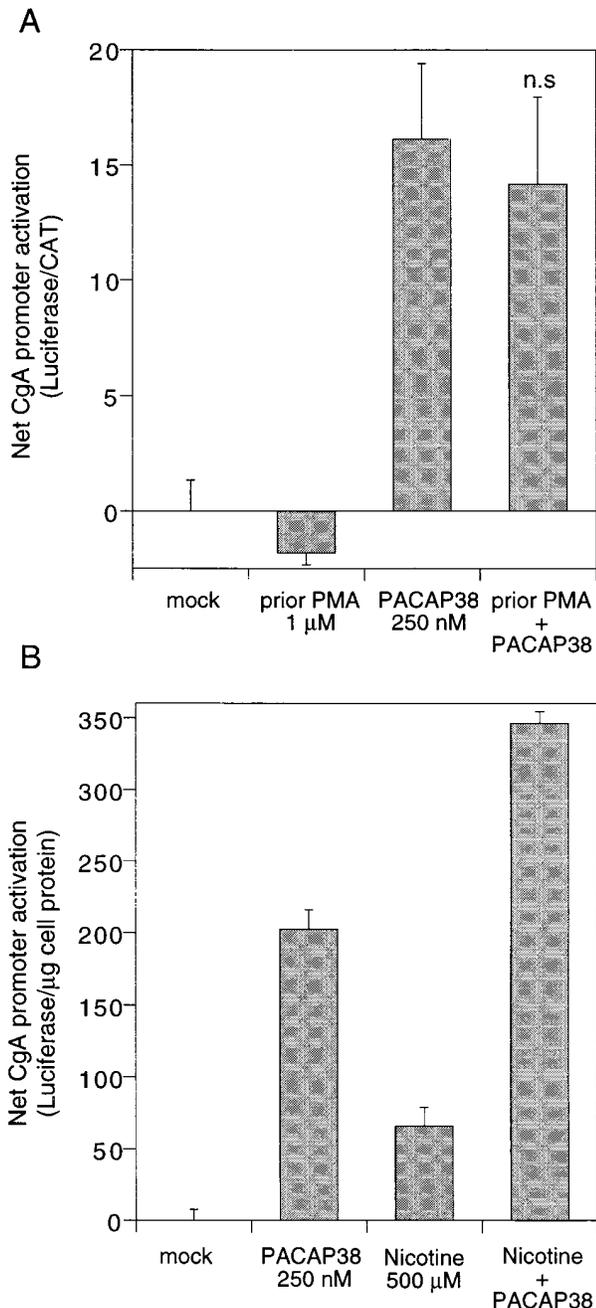


Figure 5. Transcriptional activation of the chromogranin A promoter by PACAP is not dependent on protein kinase C activation. Effect of chronic phorbol ester or acute nicotine on the response of chromogranin A promoter to PACAP. PC12 cells transfected with the 1,200-bp mouse chromogranin A promoter/luciferase reporter (pXP1200; 2.5 μ g/ml) were pretreated for 24 h with 1 μ M phorbol myristate acetate (A). After 20 h of further exposure to either 250 nM PACAP38 or vehicle (A and B), 1 mM nicotine (B), or a combination of nicotine plus PACAP38 (B), PC12 cells were assayed for luciferase activity and protein concentration. Luciferase reporter values are normalized to CAT activity resulting from cotransfected pRSV-CAT (0.5 μ g/ml) (A), or to microgram cell protein (B), representing net chromogranin A promoter activation. Values are given as the means of triplicate determinations \pm SEM of one representative experiment. *n.s.*, $P > 0.05$, as compared with the corresponding responses in the presence of PACAP38 alone (A).

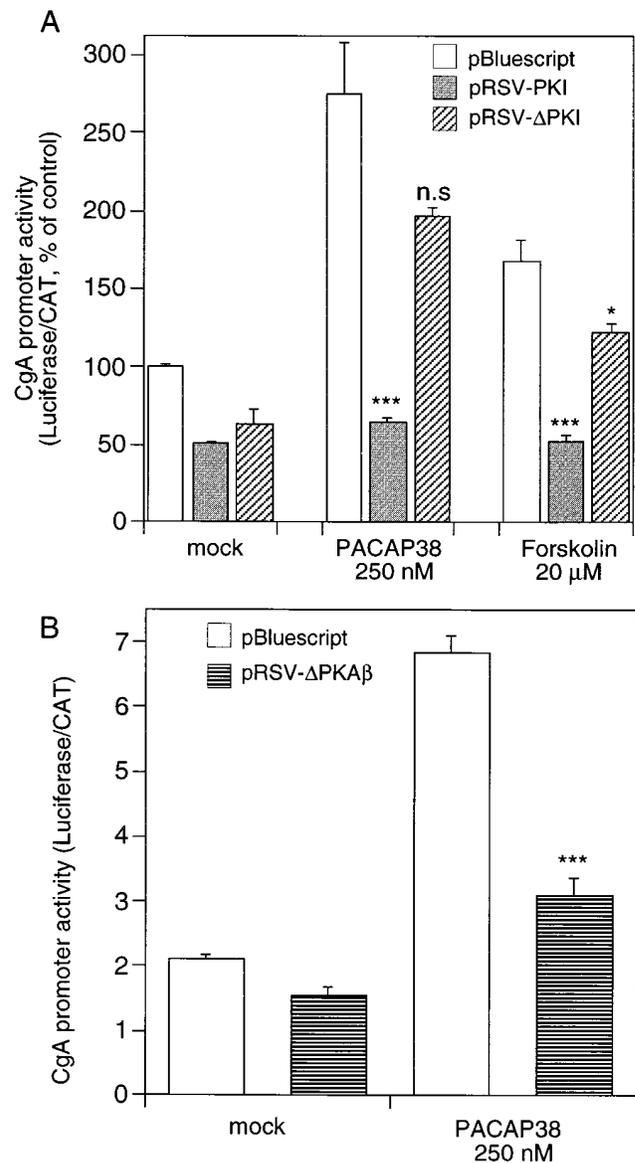


Figure 6. Involvement of protein kinase A. Effect of expression of the protein kinase A inhibitor PKI or a protein kinase A mutant catalytic β subunit on response of the chromogranin A promoter to PACAP. PC12 cells were cotransfected with the 1,200-bp mouse chromogranin A promoter/luciferase reporter (pXP1200; 2 μ g/ml) along with either: (A) plasmids encoding the protein kinase A inhibitor PKI (*pRSV-PKI*; 2 μ g/ml), an inactive PKI mutant, Δ PKI (*pRSV- Δ PKI*; 2 μ g/ml), or pBluescript (2 μ g/ml); or (B) plasmids encoding a mutant of the protein kinase A catalytic β subunit (*pRSV- Δ PKA β* ; 2 μ g/ml), or pBluescript (2 μ g/ml). Cells were subsequently incubated for 20 h in the presence or the absence of 250 nM PACAP38 (A and B) or 20 μ M forskolin (A), and processed for luciferase and CAT activities. CAT activity was assessed by cotransfection with pRSV-CAT (0.5 μ g/ml). Results are expressed as luciferase/CAT ratios normalized to mock/pBluescript control treatment (A) or as net luciferase reporter values normalized to CAT activity, representing net chromogranin A promoter activation (B). Values are given as the means of triplicate determinations \pm SEM of one representative experiment. *** $P < 0.001$; ** $P < 0.01$; and *n.s.*, $P > 0.05$, as compared with the corresponding responses in the presence of PACAP38 (A and B) or forskolin (A) alone.

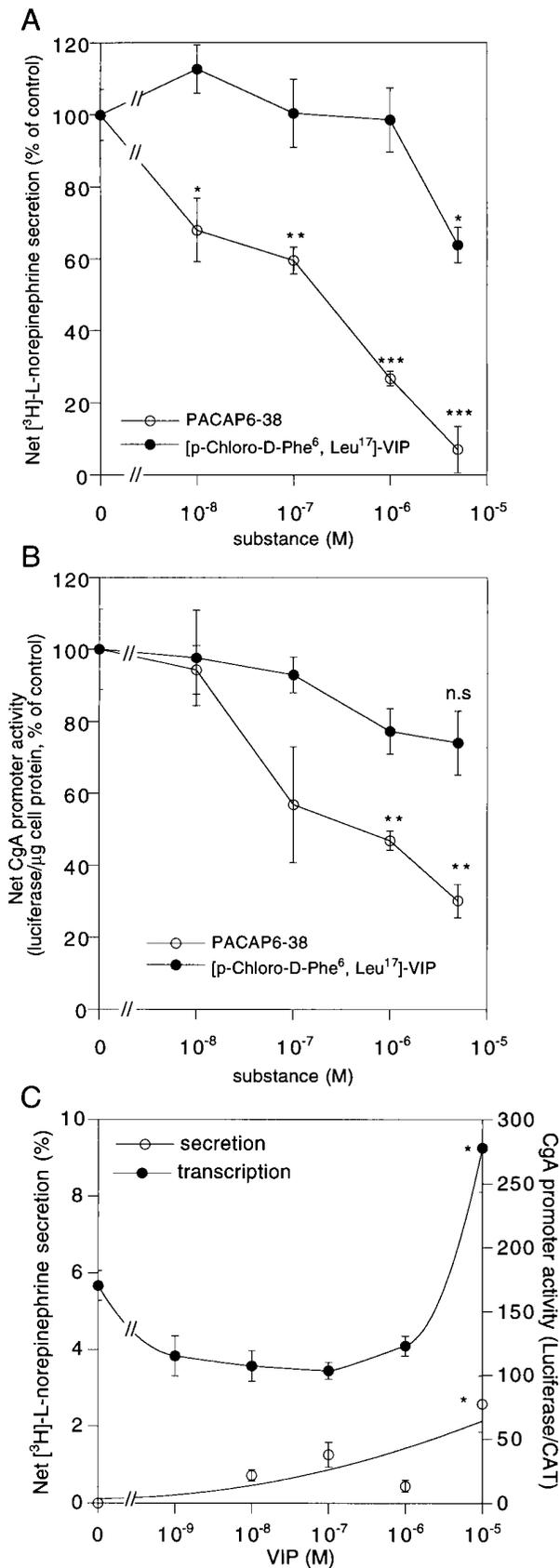


Figure 7. Involvement of the PVR1 receptor isoform in transcriptional activation of chromogranin A and stimulation of norepinephrine secretion. (A) Effect of [p-Chloro-D-Phe⁶, Leu¹⁷]-VIP or

PACAP38-induced catecholamine secretion versus PACAP-induced chromogranin A transcription was examined using the non-selective calcium channel antagonist (transition metal divalent cation) Zn²⁺, or the more selective L-type channel antagonist nifedipine. Either 100 μM ZnCl₂ or 10 μM nifedipine almost completely abolished PACAP38-evoked norepinephrine secretion (Fig. 8, A and C), but no significant inhibitory effect of either of these calcium channel blockers was observed on chromogranin A promoter activity stimulated by 250 nM PACAP38 (Fig. 8, B and D). The polycationic dye ruthenium red (50 μM), which antagonizes both L-type voltage-dependent calcium channels (60) and intracellular ryanodine receptors (61, 62) suppressed PACAP38-evoked catecholamine secretion by ~80%, but not PACAP38-induced chromogranin A trans-activation (Fig. 9, A and B).

In the same manner, the cytosolic calcium chelator BAPTA-AM (50 μM) completely abolished PACAP-induced catecholamine secretion without affecting chromogranin A trans-activation (Fig. 9, A and B). Stimulation of Ca²⁺ efflux from caffeine-sensitive Ca²⁺ stores by a low concentration of ryanodine (20 μM) did not affect either norepinephrine release or transcription of chromogranin A (Fig. 9, C and D). Similar results were obtained when ryanodine receptors were maintained in the closed state by a higher concentration of ryanodine (200 μM; data not shown). Forskolin has been reported to stimulate exocytosis in rat chromaffin cells in a

PACAP6-38 on PACAP38-induced [³H]-L-norepinephrine release. PC12 cells prelabeled with [³H]-L-norepinephrine were treated for 15 min with PACAP38 (100 nM) alone or in combination with the indicated concentrations of (a) the selective PACAP/VIP type II receptor antagonist [p-Chloro-D-Phe⁶, Leu¹⁷]-VIP, or (b) PACAP6-38, a potent PACAP/VIP type I receptor antagonist. Cells were harvested and processed for norepinephrine secretion. Results are expressed relative to the net [³H]-L-norepinephrine release obtained in the absence of antagonist (control). Values are given as the means of triplicate determinations ± SEM of one representative experiment. ***P < 0.001; **P < 0.01; *P < 0.05 as compared with mock (control) treatment. (B) Effect of [p-Chloro-D-Phe⁶, Leu¹⁷]-VIP or PACAP6-38 on PACAP38-induced chromogranin A transcriptional activation. PC12 cells transfected with the 1,200-bp mouse chromogranin A promoter/luciferase reporter (pXP1200; 2.5 μg/ml) were treated for 4 h with PACAP38 (10 nM) alone or in combination with the indicated concentrations of (a) the selective PACAP/VIP type II receptor antagonist [p-Chloro-D-Phe⁶, Leu¹⁷]-VIP, or (b) PACAP6-38, a potent PACAP/VIP type I receptor antagonist. Cells were harvested and processed for luciferase activity assay and cell protein concentration. Results are given as luciferase activity and cell protein concentration. Values are given as the means of triplicate determinations ± SEM of one representative experiment. **P < 0.01; n.s., (P > 0.05) as compared with mock (control) treatment. (C) Effect of VIP on norepinephrine secretion and chromogranin A transcription in PC12 cells. Cells transfected with the 1,200-bp mouse chromogranin A promoter/luciferase reporter (pXP1200; 2.5 μg/ml) or labeled with [³H]-L-norepinephrine, were treated for 15 min (open circles, secretion) or 20 h (black circles, transfection) with the indicated concentrations of VIP. Secretion results are expressed as percentage of net [³H]-L-norepinephrine release. Transcription results are expressed as luciferase reporter values normalized to CAT activity representing chromogranin A promoter activity. Values are given as the means of triplicate determinations ± SEM of one representative experiment. *P < 0.05 as compared with mock (control) treatment.

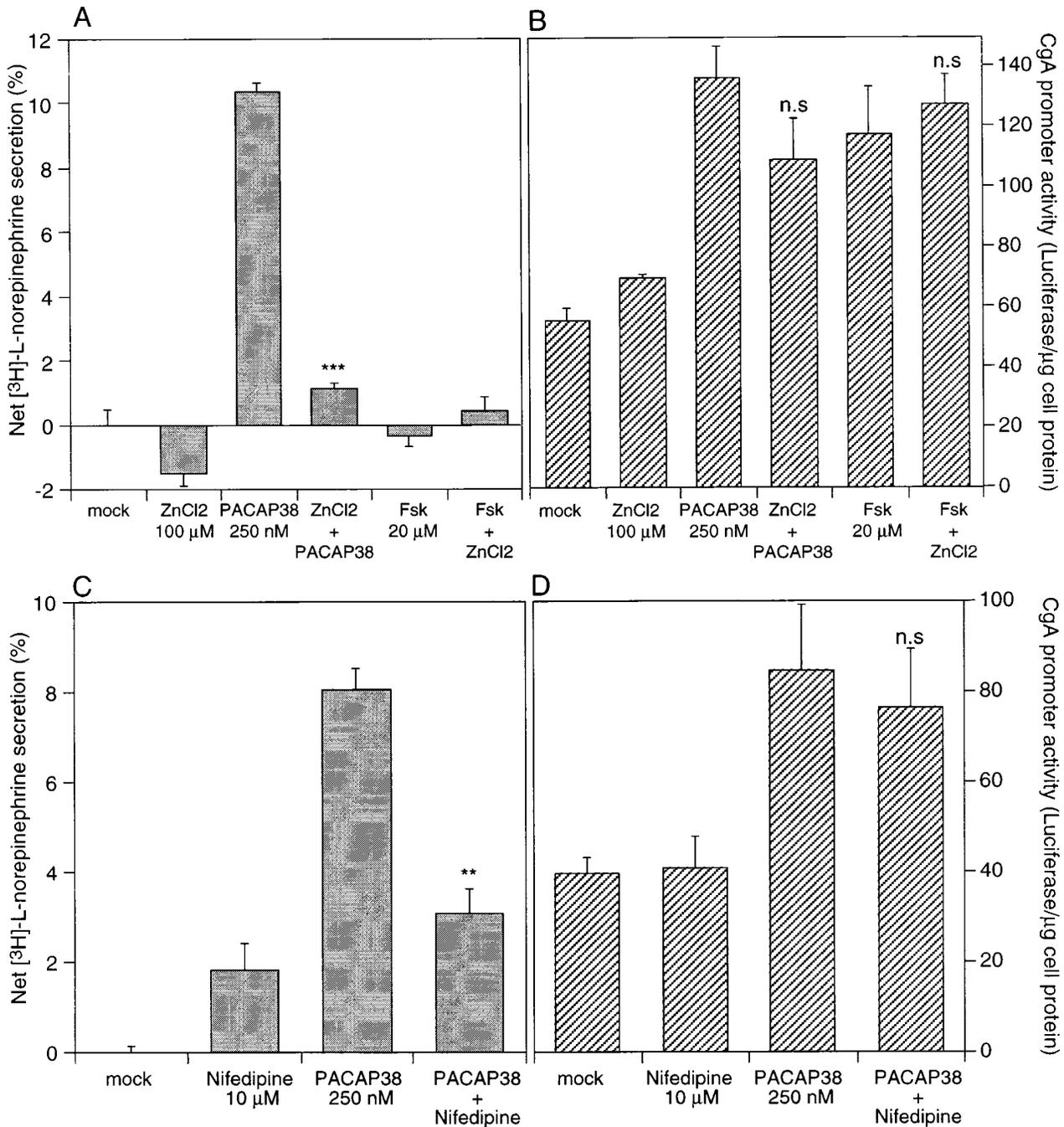


Figure 8. Influence of extracellular calcium on catecholamine secretion (A and C) or chromogranin A transcription (B and D) triggered by PACAP. PC12 cells labeled with [³H]-L-norepinephrine, or transfected with the 1,200-bp mouse chromogranin A promoter/luciferase reporter (pXP1200; 2.5 μg/ml), were preincubated in the presence or the absence of 100 μM ZnCl₂ (A and B) or 10 μM nifedipine (C and D) for 10 min (A and C, secretion) or 20 min (B and D, transcription). Cells were then treated with 250 nM PACAP38 (A–D) or 20 μM forskolin (A and B) for 15 min (A) or 20 h (B). Cells were subsequently harvested and processed for either norepinephrine secretion, or luciferase activity and cell protein concentration. Secretion data are mean ± SEM values expressed as percentage of net [³H]-L-norepinephrine release. Results are expressed as luciferase reporter values normalized to microgram cell protein, representing chromogranin A promoter activity. Values are given as the means of triplicate determinations ± SEM of one representative experiment. ****P* < 0.001; ***P* < 0.01; *n.s.*, (*P* > 0.05) as compared with the corresponding responses in the presence of PACAP38 alone.

cAMP-dependent manner (38). Although exposure of PC12 cells to forskolin (20 μM) did not stimulate catecholamine secretion (Fig. 8 A), forskolin did mimic PACAP38-induced *trans*-activation of the chromogranin A promoter, without reversal by Zn²⁺ (Fig. 8 B). Taken together, these data

suggest that PACAP38 requires extracellular Ca²⁺ entry through L-type calcium channels to stimulate catecholamine secretion. By contrast, PACAP-induced chromogranin A *trans*-activation does not seem to depend on cytosolic calcium influx.

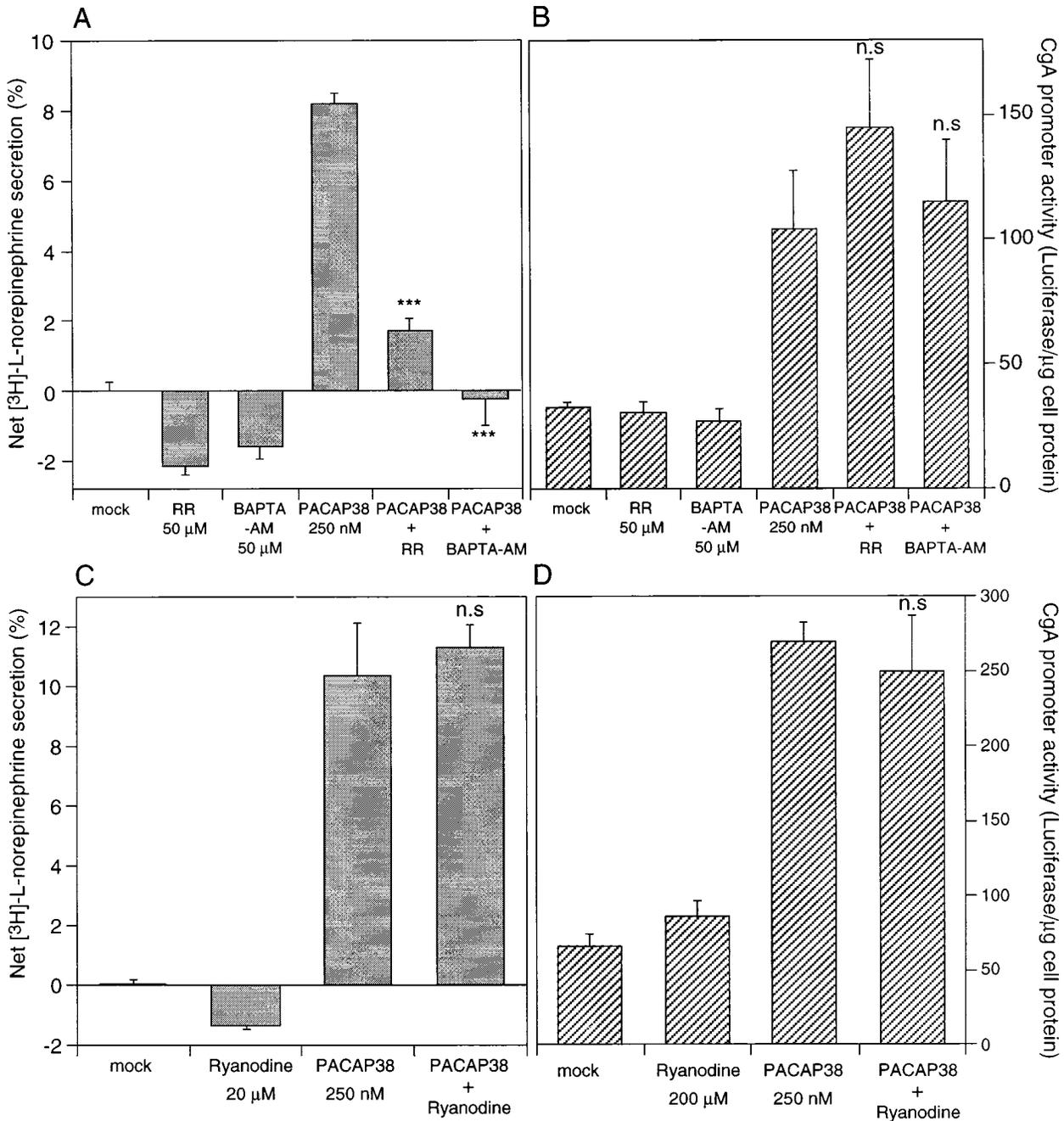


Figure 9. Influence of cytosolic calcium on catecholamine secretion and chromogranin A transcription triggered by PACAP. PC12 cells pre-labeled with [3 H]-L-norepinephrine or transfected with the 1,200-bp mouse chromogranin A promoter/luciferase reporter (pXP1200; 2.5 μ g/ml), were preincubated in the presence or the absence of 50 μ M ruthenium red (RR), 50 μ M BAPTA-AM (A and B) or 20 μ M ryanodine (C and D), for 10 min (A and C) or 20 min (B and D). Cells were then treated with vehicle or 250 nM PACAP38 for 15 min (A and C) or 20 h (B and D), and then subsequently harvested for either norepinephrine secretion or luciferase activity and cell protein concentration. Secretion results are expressed as percentage of net [3 H]-L-norepinephrine release. Transcription results are expressed as luciferase reporter values normalized to microgram cell protein, representing chromogranin A promoter activity. Values are given as the means of triplicate determinations \pm SEM of one representative experiment. *** P < 0.001; n.s. (P > 0.05) as compared with the corresponding responses in the presence of PACAP38 alone.

Discussion

During and after secretion, how do sympathoadrenal cells program resynthesis of their just-secreted components? Does the same physiologic signal that evokes secretion also trigger resynthesis of the just-secreted peptides and catecholamines? If

so, what is the mechanism of such stimulus–transcription coupling? Several recent reports provide evidence that PACAP is a potent peptidergic neurotransmitter governing both secretion and synthesis of catecholamines in chromaffin cells (33–35). Moreover, a distinct role for PACAP in addition to that of the cholinergic transmitter acetylcholine has been suggested

(38). However, mechanisms whereby PACAP might influence biosynthesis of just-secreted proteins and neuropeptides of the chromaffin cell secretory granule have not yet been elucidated (36). Therefore, we used PC12 cells to examine how PACAP might influence both catecholamine secretory activity and chromogranin A biosynthesis.

An important prerequisite for the significance of any study of transcription is that expression of the endogenous (chromosomal) gene be activated by the stimulus under investigation. Here we found that PACAP augmented chromogranin A mRNA by approximately four- to fivefold in PC12 cells (Fig. 1). Approximately seven- to eightfold *trans*-activation of a transfected 1,200-bp mouse chromogranin A promoter/luciferase reporter construct provided further evidence for transcriptional activation by PACAP. In addition, deletion mutation studies suggested that the chromogranin A proximal promoter is entirely necessary to confer the PACAP response onto the chromogranin A gene (Fig. 3 A). The transcriptional response of the transfected chromogranin A promoter to PACAP showed a decline after prolonged stimulation (24–48 h) with PACAP (Fig. 2 C). This observation may indicate desensitization (uncoupling) of the G protein-coupled PACAP receptor by either phosphorylation or sequestration, as suggested by general patterns in other agonist-induced G protein-coupled receptor desensitizations (63).

A series of chromogranin A promoter 5' deletion mutant/reporter constructs revealed several regulatory domains in *cis* responding to PACAP stimulation (Fig. 3 A). Promoter domains whose deletion decreased the PACAP response were both distal (–1,200 to –821 bp) and proximal (–77 to –61 bp), while a domain from –181 to –77 bp conferred a negative response to PACAP (i.e., its deletion increased PACAP response). This suggests that the chromogranin A CRE element, located in the –77 to –61 bp segment ([–71 bp]5'-TGACG-TAA-3'[-64 bp]) may have a substantial role in mediating the PACAP response. CRE domains also seem to mediate PACAP activation of gene expression in other neuroendocrine cell types, including prolactin expression in GH3 somatotropes (64) or POMC expression in AtT20 corticotropes (65). The CRE motif of the *c-fos* promoter is also PACAP-responsive in PC12 cells (66).

Since the CRE domain in the chromogranin A promoter is conserved across species (45, 67–69) and responds to cAMP (46), we evaluated the role of this CRE in response to PACAP, a cAMP-generating stimulus. The results of three independent experimental approaches gave evidence that the CRE domain in the chromogranin A promoter is entirely necessary and, to some extent, sufficient to account for the PACAP response. First, both promoter deletion past the CRE (Fig. 3 A) and dense site-directed point mutagenesis of the CRE (mutants M9, M12, M13, M15, and M16; Fig. 3 B) markedly diminished PACAP effects, while mutation M14 (which leads to a consensus CRE domain) enhanced the PACAP response (Fig. 3 B). Second, both the chromogranin A CRE (TGACGTAA) and a consensus CRE (TGACGTCA) conferred the PACAP response onto a heterologous (thymidine kinase), previously unresponsive promoter (Fig. 3 C). Finally, transfection of the CREB DNA-binding domain mutant KCREB blunted PACAP activation of two CRE-containing (pXP77 and pXP1200) chromogranin A promoter constructs (Fig. 4 A), and inhibited direct cAMP activation of an isolated CRE element (Fig. 4 B).

Depending upon the particular postreceptor G protein isoform activated, PACAP stimulation may exhibit multifunctional downstream signaling characteristics, including activation of the adenylyl cyclase/cAMP pathway, the phospholipase C/phosphoinositide/Ca²⁺ pathway, or the Ras/Raf pathway (8, 66, 70). We found that PACAP stimulates both transcriptional activation of chromogranin A and catecholamine secretion (Fig. 2, A and B), but the signaling pathways employed by these two responses were quite distinct. However, both transcription and secretion are mediated by the same PACAP/VIP type I (PVR1) receptor isoform (Fig. 7, A–C), consistent with the high abundance of this isoform in the adrenal medulla (40).

In chromaffin cells, PACAP has been reported to stimulate both cAMP and phosphoinositide/Ca²⁺ signaling pathways (70). Isobe et al. (71) found that the biosynthetic responses of tyrosine hydroxylase and dopamine β-hydroxylase to PACAP are mediated by both cAMP and protein kinase C signaling pathways. By genetic inactivation of protein kinase A with the dominant inhibitory mutant PKI or a dominant negative catalytic subunit (ΔPKAβ), we demonstrated that PACAP signals to chromogranin A gene expression largely if not exclusively by the adenylyl cyclase/cAMP/protein kinase A pathway (Fig. 6, A and B). Consistent with this picture, a role of protein kinase C (i.e., the phospholipase C/phosphoinositide/Ca²⁺ pathway) could not be documented: downregulation of protein kinase C by phorbol myristate acetate did not affect PACAP-induced chromogranin A *trans*-activation (Fig. 5 A) or PACAP-evoked catecholamine secretion (data not shown). Furthermore, approximately additive transcriptional effects of PACAP plus nicotine, a protein kinase C-dependent chromogranin A gene *trans*-activator (57), suggested different signaling pathways for these two secretory agonists (Fig. 5 B). Finally, PACAP effects on transcription were mimicked by a direct activator of adenylyl cyclase, forskolin (Fig. 6, A and B), while forskolin had no effect on catecholamine release in PC12 cells (Fig. 8 A) indicating that PACAP uses a cAMP-independent pathway for its secretory effect in these cells. The role of cAMP in mediating PACAP's effects on secretion is not completely understood. Forskolin may trigger exocytosis in rat chromaffin cells (38), but has no secretory effect on porcine chromaffin cells (71) or rat PC12 cells (57).

In bovine and porcine chromaffin cells, a key step in PACAP-evoked catecholamine secretion is extracellular calcium influx, most likely through L-type voltage-gated calcium channels (33, 34) or as yet incompletely characterized, Zn²⁺-sensitive calcium channels in rat chromaffin cells (38). We found that PACAP-triggered catecholamine release was inhibited by not only the nonselective calcium channel blocker Zn²⁺ (38), but also the dihydropyridine nifedipine and ruthenium red, which more selectively antagonize L-type voltage gated calcium channels (Fig. 8, A and C; Fig. 9 A). Thus far, the mechanism by which PVR1 receptor activation opens such calcium channels is not completely documented. One hypothesis is direct opening of L-type calcium channels by the G protein-coupled receptor (e.g., by a G_{sa}), as suggested for activation of calcium influx by the β1-adrenoreceptor in cardiomyocytes (72). Protein kinase A might also phosphorylate and thereby activate calcium channels (73), though we found no effect of the cAMP pathway on catecholamine secretion from PC12 cells (57).

In contrast to secretion, blockade of extracellular calcium influx by Zn²⁺ or nifedipine had no effect on transcriptional

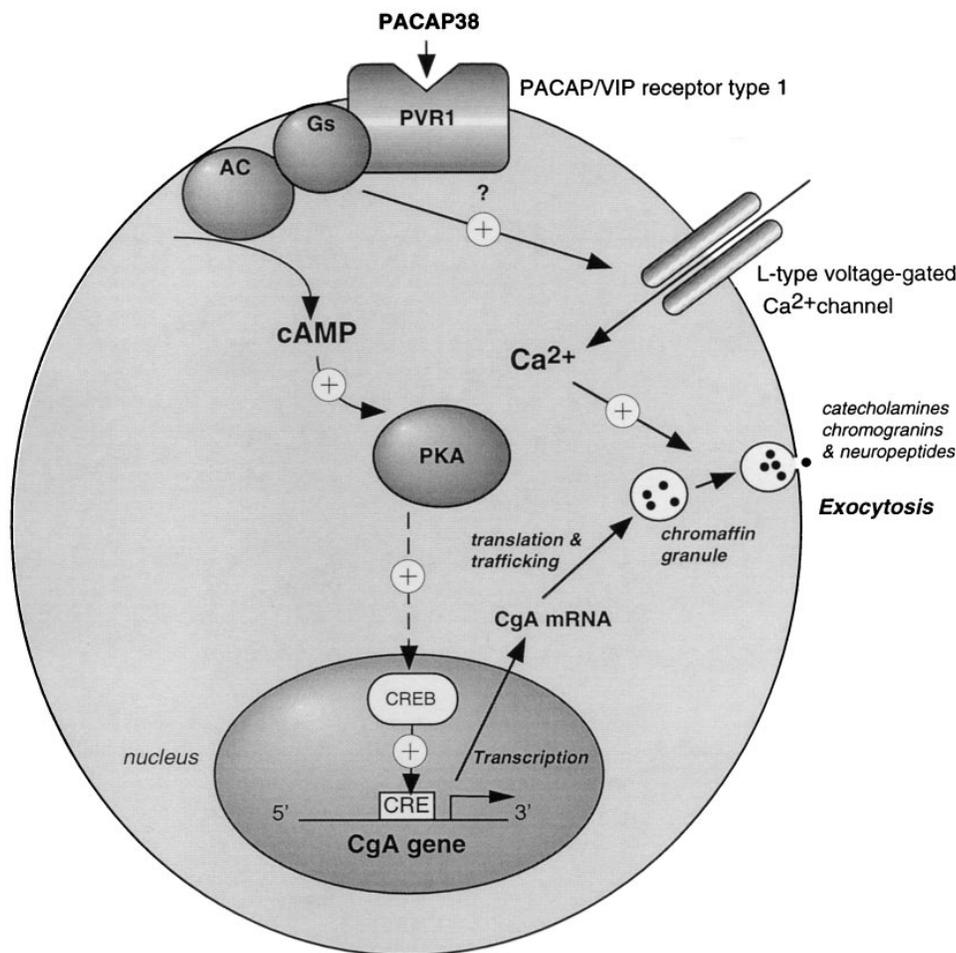


Figure 10. Putative signal transduction pathways underlying PACAP-induced catecholamine release and chromogranin A transcription in PC12 cells: results suggested by the current experiments. CRE: cAMP response element (here: [-71 bp]5'-TGACGTAA-39[-64 bp]). CREB, homodimeric CRE-binding/trans-activating protein; AC, adenylyl cyclase; PKA, cAMP-dependent protein kinase A. CgA: chromogranin A; Gs, stimulatory heterotrimeric G protein; PACAP38, 38 amino acid active form of PACAP; (+) stimulation or activation; (?) pathway not clearly established.

activation of chromogranin A by PACAP (Fig. 8, B and D). While chelation of cytosolic calcium abolished PACAP-evoked secretion (Fig. 9 A), transcription was unaffected (Fig. 9 B). In bovine chromaffin cells, PACAP has been shown to induce Ca^{2+} release mainly from caffeine-sensitive intracellular stores (34). Here we found that maintaining intracellular caffeine-sensitive calcium channels (i.e., ryanodine receptors) in an open state by low concentration of ryanodine or in a closed conformation by high concentration of this alkaloid did not affect the PACAP responses of secretion or transcription (Fig. 9, C and D). It is of note that ruthenium red, in addition to its blocking effects on L-type calcium channels, is also (and perhaps more importantly) a potent inhibitor of intracellular calcium release from ryanodine-sensitive stores (61, 62). Since ruthenium red did not impair PACAP-evoked transcription, calcium release from intracellular stores is not likely to be crucial in mediating either catecholamine secretion or transcriptional activation by PACAP.

In conclusion, we propose divergent signal transduction schemes for secretory stimulation and transcriptional activation of the chromogranin A gene by PACAP (Fig. 10). PACAP mediates both secretion and transcription through a PACAP/VIP type I (PVR1) receptor, but with quite different postreceptor signaling pathways. PACAP signals to chromogranin A transcription through the CRE domain in *cis*, and through protein kinase A and the transcription factor CREB

in *trans*. Whereas extracellular Ca^{2+} influx mediates catecholamine secretion, PACAP-induced chromogranin A transcriptional activation is a calcium-independent process.

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References

1. Miyata, A., L. Jiang, H.H. Stibbs, and A. Arimura. 1992. Chemical characterization of vasoactive intestinal polypeptide-like immunoreactivity in ovine hypothalamus and intestine. *Regul. Pept.* 38:145-154.
2. Arimura, A. 1992. Pituitary adenylate cyclase activating polypeptide (PACAP): discovery and current status of research. *Regul. Pept.* 37:287-303.
3. Lutz, E.M., W.J. Sheward, K.M. West, J.A. Morrow, G. Fink, and A.J. Harmar. 1993. The VIP2 receptor: molecular characterisation of a cDNA encoding a novel receptor for vasoactive intestinal peptide. *FEBS Lett.* 334:3-8.
4. Pisegna, J.R., and S.A. Wank. 1993. Molecular cloning and functional expression of the pituitary adenylate cyclase-activating polypeptide type I receptor. *Proc. Natl. Acad. Sci. USA.* 90:6345-6349.
5. Svoboda, M., M. Tastenoy, E. Ciccarelli, M. Stievenart, and J. Chris-

- tophe. 1993. Cloning of a splice variant of the pituitary adenylate cyclase-activating polypeptide (PACAP) type I receptor. *Biochem. Biophys. Res. Commun.* 195:881–888.
6. Spengler, D., C. Waerber, C. Pantaloni, F. Holsboer, J. Bockaert, P.H. Seeburg, and L. Journot. 1993. Differential signal transduction by five splice variants of the PACAP receptor. *Nature*. 365:170–175.
7. Rawlings, S.R., and M. Hezareh. 1996. Pituitary adenylate cyclase-activating polypeptide (PACAP) and PACAP/vasoactive intestinal polypeptide receptors: actions on the anterior pituitary gland. *Endocr. Rev.* 17:4–29.
8. Zhong, Y. 1995. Mediation of PACAP-like neuropeptide transmission by coactivation of Ras/Raf and cAMP signal transduction pathways in *Drosophila*. *Nature*. 375:588–592.
9. Yada, T., M. Sakurada, K. Ihida, M. Nakata, F. Murata, A. Arimura, and M. Kikuchi. 1994. Pituitary adenylate cyclase activating polypeptide is an extraordinarily potent intra-pancreatic regulator of insulin secretion from islet beta-cells. *J. Biol. Chem.* 269:1290–1293.
10. Hashimoto, H., T. Ishihara, R. Shigemoto, K. Mori, and S. Nagata. 1993. Molecular cloning and tissue distribution of a receptor for pituitary adenylate cyclase-activating polypeptide. *Neuron*. 11:333–342.
11. Arimura, A., and S. Shioda. 1995. Pituitary adenylate cyclase activating polypeptide (PACAP) and its receptors: neuroendocrine and endocrine interaction. *Front. Neuroendocrinol.* 16:53–88.
12. O'Connor, D.T. 1983. Chromogranin: widespread immunoreactivity in polypeptide hormone producing tissues and in serum. *Regul. Pept.* 6:263–280.
13. Simon, J.P., and D. Aunis. 1989. Biochemistry of the chromogranin A protein family. *Biochem. J.* 262:1–13.
14. Winkler, H. 1993. The adrenal chromaffin granule: a model for large dense core vesicles of endocrine and nervous tissue. *J. Anat.* 183:237–252.
15. Daniels, A.J., R.J. Williams, and P.E. Wright. 1978. The character of the stored molecules in chromaffin granules of the adrenal medulla: a nuclear magnetic resonance study. *Neuroscience*. 3:573–585.
16. Yoo, S.H., and J.P. Albanesi. 1991. High capacity, low affinity Ca^{2+} binding of chromogranin A. Relationship between the pH-induced conformational change and Ca^{2+} binding property. *J. Biol. Chem.* 266:7740–7745.
17. Videen, J.S., M.S. Mezger, Y.M. Chang, and D.T. O'Connor. 1992. Calcium and catecholamine interactions with adrenal chromogranins. Comparison of driving forces in binding and aggregation. *J. Biol. Chem.* 267:3066–3073.
18. Seidah, N.G., G.N. Hendy, J. Hamelin, J. Paquin, C. Lazure, K.M. Metters, J. Rossier, and M. Chretien. 1987. Chromogranin A can act as a reversible processing enzyme inhibitor. Evidence from the inhibition of the IRCM-serine protease 1 cleavage of pro-enkephalin and ACTH at pairs of basic amino acids. *FEBS Lett.* 211:144–150.
19. Parmer, R.J., X.P. Xi, H.J. Wu, L.J. Helman, and L.N. Petz. 1993. Secretory protein traffic. Chromogranin A contains a dominant targeting signal for the regulated pathway. *J. Clin. Invest.* 92:1042–1054.
20. Iacangelo, A.L., and L.E. Eiden. 1995. Chromogranin A: current status as a precursor for bioactive peptides and a granulogenic/sorting factor in the regulated secretory pathway. *Regul. Pept.* 58:65–88.
21. Tatamoto, K., S. Efendic, V. Mutt, G. Makk, G.J. Feistner, and J.D. Barchas. 1986. Pancreastatin, a novel pancreatic peptide that inhibits insulin secretion. *Nature*. 324:476–478.
22. Taupenot, L., J. Ciesielski-Treska, G. Ulrich, S. Chasserot-Golaz, D. Aunis, and M.F. Bader. 1996. Chromogranin A triggers a phenotypic transformation and the generation of nitric oxide in brain microglial cells. *Neuroscience*. 72:377–389.
23. Fasciotto, B.H., C.A. Trauss, G.H. Greeley, and D.V. Cohn. 1993. Parastatin (porcine chromogranin A347-419), a novel chromogranin A-derived peptide, inhibits parathyroid cell secretion. *Endocrinology*. 133:461–466.
24. Simon, J.P., M.F. Bader, and D. Aunis. 1988. Secretion from chromaffin cells is controlled by chromogranin A-derived peptides. *Proc. Natl. Acad. Sci. USA*. 85:1712–1716.
25. Aardal, S., K.B. Helle, S. Elsayed, R.K. Reed, and G. Serck-Hanssen. 1993. Vasostatin, comprising the N-terminal domain of chromogranin A, suppress tension in isolated human blood vessel segments. *J. Neuroendocrinol.* 5:405–412.
26. Mahata, S.K., D.T. O'Connor, M. Mahata, S.H. Yoo, L. Taupenot, H. Wu, B.M. Gill, and R.J. Parmer. 1997. Novel autocrine feedback control of catecholamine release: a discrete chromogranin A fragment is a non-competitive nicotinic cholinergic antagonist. *J. Clin. Invest.* 100:1623–1633.
27. Tischler, A.S., R.L. Perlman, D. Costopoulos, and J. Horwitz. 1985. Vasoactive intestinal peptide increases tyrosine hydroxylase activity in normal and neoplastic rat chromaffin cell cultures. *Neurosci. Lett.* 61:141–146.
28. Houchi, H., M. Oka, M. Misbahuddin, K. Morita, and A. Nakanishi. 1987. Stimulation by vasoactive intestinal polypeptide of catecholamine synthesis in isolated bovine adrenal chromaffin cells. Possible involvement of protein kinase C. *Biochem. Pharmacol.* 36:1551–1554.
29. Malhotra, R.K., T.D. Wakade, and A.R. Wakade. 1989. Cross-communication between acetylcholine and VIP in controlling catecholamine secretion by affecting cAMP, inositol triphosphate, protein kinase C, and calcium in rat adrenal medulla. *J. Neurosci.* 9:4150–4157.
30. Malhotra, R.K., and A.R. Wakade. 1987. Non-cholinergic component of rat splanchnic nerves predominates at low neuronal activity and is eliminated by naloxone. *J. Physiol. (Lond)*. 383:639–652.
31. Wakade, A.R. 1988. Noncholinergic transmitter(s) maintains secretion of catecholamines from rat adrenal medulla for several hours of continuous stimulation of splanchnic neurons. *J. Neurochem.* 50:1302–1308.
32. Watanabe, T., Y. Masuo, H. Matsumoto, N. Suzuki, T. Ohtaki, Y. Masuda, C. Kitada, M. Tsuda, and M. Fujino. 1992. Pituitary adenylate cyclase activating polypeptide provokes cultured rat chromaffin cells to secrete adrenaline. *Biochem. Biophys. Res. Commun.* 182:403–411.
33. Isobe, K., T. Nakai, and Y. Takuwa. 1993. Ca^{2+} -dependent stimulatory effect of pituitary adenylate cyclase-activating polypeptide on catecholamine secretion from cultured porcine adrenal medullary chromaffin cells. *Endocrinology*. 132:1757–1765.
34. Tanaka, K., I. Shibuya, T. Nagamoto, H. Yamashita, and T. Kanno. 1996. Pituitary adenylate cyclase-activating polypeptide causes rapid Ca^{2+} release from intracellular stores and long lasting Ca^{2+} influx mediated by Na^{+} influx-dependent membrane depolarization in bovine adrenal chromaffin cells. *Endocrinology*. 137:956–966.
35. Marley, P.D., C.Y. Cheung, K.A. Thomson, and R. Murphy. 1996. Activation of tyrosine hydroxylase by pituitary adenylate cyclase-activating polypeptide (PACAP-27) in bovine adrenal chromaffin cells. *J. Auton. Nerv. Syst.* 60:141–146.
36. Babinski, K., V. Bodart, M. Roy, A. De Lean, and H. Ong. 1996. Pituitary adenylate-cyclase activating polypeptide (PACAP) evokes long-lasting secretion and de novo biosynthesis of bovine adrenal medullary neuropeptides. *Neuropeptides*. 30:572–582.
37. Rius, R.A., A. Guidotti, and E. Costa. 1994. Pituitary adenylate cyclase activating polypeptide (PACAP) potentially enhances tyrosine hydroxylase (TH) expression in adrenal chromaffin cells. *Life Sci.* 54:1735–1743.
38. Przywara, D.A., X. Guo, M.L. Angelilli, T.D. Wakade, and A.R. Wakade. 1996. A non-cholinergic transmitter, pituitary adenylate cyclase-activating polypeptide, utilizes a novel mechanism to evoke catecholamine secretion in rat adrenal chromaffin cells. *J. Biol. Chem.* 271:10545–10550.
39. Tabarin, A., D. Chen, R. Hakanson, and F. Sundler. 1994. Pituitary adenylate cyclase-activating peptide in the adrenal gland of mammals: distribution, characterization and responses to drugs. *Neuroendocrinology*. 59:113–119.
40. Moller, K., and F. Sundler. 1996. Expression of pituitary adenylate cyclase activating peptide (PACAP) and PACAP type I receptors in the rat adrenal medulla. *Regul. Pept.* 63:129–139.
41. Boksa, P., and B.G. Livett. 1984. Desensitization to nicotinic cholinergic agonists and K^{+} , agents that stimulate catecholamine secretion, in isolated adrenal chromaffin cells. *J. Neurochem.* 42:607–617.
42. Lukas, R.J., and M. Bencherif. 1992. Heterogeneity and regulation of nicotinic acetylcholine receptors. *Int. Rev. Neurobiol.* 34:25–131.
43. Greene, L.A., and A.S. Tischler. 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA*. 73:2424–2428.
44. Mahata, M., S.K. Mahata, R.J. Parmer, and D.T. O'Connor. 1996. Vesicular monoamine transport inhibitors. Novel action at calcium channels to prevent catecholamine secretion. *Hypertension (Dallas)*. 28:414–420.
45. Wu, H., D.J. Rozansky, N.J. Webster, and D.T. O'Connor. 1994. Cell type-specific gene expression in the neuroendocrine system. A neuroendocrine-specific regulatory element in the promoter of chromogranin A, a ubiquitous secretory granule core protein. *J. Clin. Invest.* 94:118–129.
46. Wu, H., S.K. Mahata, M. Mahata, N.J. Webster, R.J. Parmer, and D.T. O'Connor. 1995. A functional cyclic AMP response element plays a crucial role in neuroendocrine cell type-specific expression of the secretory granule protein chromogranin A. *J. Clin. Invest.* 96:568–578.
47. Roesler, W.J., G.R. Vandenbark, and R.W. Hanson. 1988. Cyclic AMP and the induction of eukaryotic gene transcription. *J. Biol. Chem.* 263:9063–9066.
48. Walton, K.M., R.P. Rehfuss, J.C. Chrivia, J.E. Lochner, and R.H. Goodman. 1992. A dominant repressor of cyclic adenosine 3',5'-monophosphate (cAMP)-regulated enhancer-binding protein activity inhibits the cAMP-mediated induction of the somatostatin promoter in vivo. *Mol. Endocrinol.* 6:647–655.
49. Day, R.N., J.A. Walder, and R.A. Maurer. 1989. A protein kinase inhibitor gene reduces both basal and multihormone-stimulated prolactin gene transcription. *J. Biol. Chem.* 264:431–436.
50. Maurer, R.A. 1989. Both isoforms of the cAMP-dependent protein kinase catalytic subunit can activate transcription of the prolactin gene. *J. Biol. Chem.* 264:6870–6873.
51. Gorman, C.M., L.F. Moffat, and B.H. Howard. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* 2:1044–1051.
52. Tang, K., H. Wu, S.K. Mahata, L. Taupenot, D.J. Rozansky, R.J. Parmer, and D.T. O'Connor. 1996. Stimulus-transcription coupling in pheochromocytoma cells. Promoter region-specific activation of chromogranin A biosynthesis. *J. Biol. Chem.* 271:28382–28390.
53. de Wet, J.R., K.V. Wood, M. DeLuca, D.R. Helinski, and S. Subramani. 1987. Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell. Biol.* 7:725–737.
54. Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye bind-

ing. *Anal. Biochem.* 72:248–254.

55. Parmer, R.J., A.H. Koop, M.T. Handa, and D.T. O'Connor. 1989. Molecular cloning of chromogranin A from rat pheochromocytoma cells. *Hypertension (Dallas)*. 14:435–444.
56. Hasel, K.W., and J.G. Sutcliffe. 1990. Nucleotide sequence of a cDNA coding for mouse cyclophilin. *Nucleic Acids Research*. 18:4019.
57. Tang, K., H. Wu, B.M. Gill, S.K. Mahata, M. Mahata, and D.T. O'Connor. 1997. Stimulus coupling to transcription versus secretion in pheochromocytoma cells: convergent and divergent signal transduction pathways, and the crucial role for route of cytosolic calcium entry and protein kinase C. *J. Clin. Invest.* 100:1180–1192.
58. Robberecht, P., P. Gourlet, P. De Neef, M.C. Woussen-Colle, M.C. Vandermeers-Piret, A. Vandermeers, and J. Christophe. 1992. Structural requirements for the occupancy of pituitary adenylate-cyclase-activating-peptide (PACAP) receptors and adenylate cyclase activation in human neuroblastoma NB-OK-1 cell membranes. Discovery of PACAP (6-38) as a potent antagonist. *Eur. J. Biochem.* 207:239–246.
59. Pandol, S.J., K. Dharmasathaphorn, M.S. Schoeffield, W. Vale, and J. Rivier. 1986. Vasoactive intestinal peptide receptor antagonist [4Cl-D-Phe6, Leu17] VIP. *Am. J. Physiol.* 250:G553–G557.
60. Gomis, A., L.M. Gutierrez, F. Sala, S. Viniegra, and J.A. Reig. 1994. Ruthenium red inhibits selectively chromaffin cell calcium channels. *Biochem. Pharmacol.* 47:225–231.
61. Volpe, P., G. Salviati, and A. Chu. 1986. Calcium-gated calcium channels in sarcoplasmic reticulum of rabbit skinned skeletal muscle fibers. *J. Gen. Physiol.* 87:289–303.
62. Conley, E.C. 1996. Intracellular ligand-gated channels. Harcourt Brace & Co., London. 77 pp.
63. Chuang, T.T., L. Iacovelli, M. Sallèse, and A. De Blasi. 1996. G protein-coupled receptors: heterologous regulation of homologous desensitization and its implications. *Trends Pharmacol. Sci.* 17:416–421.
64. Coleman, D.T., X. Chen, M. Sassaroli, and C. Bancroft. 1996. Pituitary adenylate cyclase-activating polypeptide regulates prolactin promoter activity via a protein kinase A-mediated pathway that is independent of the transcriptional pathway employed by thyrotropin-releasing hormone. *Endocrinology*. 137:1276–1285.
65. Boutillier, A.L., D. Monnier, B. Koch, and J.P. Loeffler. 1994. Pituitary adenyl cyclase-activating peptide: a hypophysiotropic factor that stimulates proopiomelanocortin gene transcription, and proopiomelanocortin-derived peptide secretion in corticotropic cells. *Neuroendocrinology*. 60:493–502.
66. Schadlow, V.C., N. Barzilai, and P.J. Deutsch. 1992. Regulation of gene expression in PC12 cells via an activator of dual second messengers: pituitary adenylate cyclase activating polypeptide. *Mol. Biol. Cell.* 3:941–951.
67. Iacangelo, A.L., M. Grimes, and L.E. Eiden. 1991. The bovine chromogranin A gene: structural basis for hormone regulation and generation of biologically active peptides. *Mol. Endocrinol.* 5:1651–1660.
68. Rozansky, D.J., H. Wu, K. Tang, R.J. Parmer, and D.T. O'Connor. 1994. Glucocorticoid activation of chromogranin A gene expression. Identification and characterization of a novel glucocorticoid response element. *J. Clin. Invest.* 94:2357–2368.
69. Moulant, A.J., S. Bevan, J.H. White, and G.N. Hendy. 1994. Human chromogranin A gene. Molecular cloning, structural analysis, and neuroendocrine cell-specific expression. *J. Biol. Chem.* 269:6918–6926.
70. Deutsch, P.J., and Y. Sun. 1992. The 38-amino acid form of pituitary adenylate cyclase-activating polypeptide stimulates dual signaling cascades in PC12 cells and promotes neurite outgrowth. *J. Biol. Chem.* 267:5108–5113.
71. Isobe, K., N. Yukimasa, T. Nakai, and Y. Takuwa. 1996. Pituitary adenylate cyclase-activating polypeptide induces gene expression of the catecholamine synthesizing enzymes, tyrosine hydroxylase and dopamine beta hydroxylase, through 3',5'-cyclic adenosine monophosphate- and protein kinase C-dependent mechanisms in cultured porcine adrenal medullary chromaffin cells. *Neuropeptides*. 30:167–175.
72. Castellano, M., and M. Böhm. 1997. The cardiac β -adrenoreceptor-mediated signaling pathway and its alteration in hypertensive heart disease. *Hypertension (Dallas)*. 29:715–722.
73. Adelman, J.P. 1995. Proteins that interact with the pore-forming subunits of voltage-gated ion channels. *Curr. Opin. Neurobiol.* 5:286–295.