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Pituitary Adenylate Cyclase-Activating Polypeptide Stimulates Secretoneurin Release and Secretogranin II Gene Transcription in Bovine Adrenochromaffin Cells through Multiple Signaling Pathways and Increased Binding of Pre-Existing Activator Protein-1-Like Transcription Factors

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ABSTRACT

Secretoneurin (SN) is a novel bioactive peptide that derives from the neuroendocrine protein secretogranin II (SgII) by proteolytic processing and participates in neuro-immune communication. The neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP-38) dose-dependently stimulates ($EC_{50} \sim 3$ nM) SN release (up to 4-fold) and SgII gene expression (up to 60-fold) in cultured bovine adrenochromaffin cells. The effect of PACAP on both SN secretion and SgII mRNA levels is rapid and long lasting. We analyzed in this neuroendocrine cell model the transduction pathways involved in both SN secretion and SgII gene transcription in response to PACAP. The cytosolic calcium chelator BAPTA-AM and the nonselective calcium channel antagonist $NiCl_2$ equally inhibited both secretion of the peptide and transcription of the SgII gene, indicating a major contribution of calcium influx in PACAP-induced SN biosynthesis and release in chromaffin cells. Inhibition of protein kinase A (PKA) or C (PKC) also reduced PACAP-evoked SN release but did not alter the stimulatory effect of PACAP on SgII mRNA levels. Conversely, application of mitogen-activated protein kinase inhibitors suppressed PACAP-induced SgII gene expression. The effect of PACAP on SgII mRNA levels, like the effect of the PKC stimulator 12-*O*-tetradecanoylphorbol-13-acetate (TPA), was not affected by cycloheximide, whereas the effects of the PKA stimulator forskolin or cell-depolarization by high K^+ were significantly reduced by the protein synthesis inhibitor. PACAP and TPA both increased the binding activity of the SgII cAMP response element to *trans*-acting factors present in chromaffin cell nuclear extracts, which are recognized by antibodies to activator protein-1-related proteins. These data indicate that SN biosynthesis is regulated by PACAP in chromaffin cells through complex signaling cascades, suggesting that SN may play a function during *trans*-synaptic stimulation of the adrenal medulla.

Secretoneurin (SN) is a novel 33-amino-acid peptide originally isolated from the frog brain (Vaudry and Conlon, 1991) and subsequently identified in a variety of mammalian tissues (Kirchmair et al., 1993). SN has been shown to stimulate dopamine release in the striatum (Agneter et al., 1995) and to participate in neuro-immune communication owing to its chemotactic activity toward monocytes in inflammatory sites (Dunzendorfer et al., 1998). Binding studies suggest the existence of specific G protein-coupled receptors on the surface of monocytes that recognize SN and activate PKC (Kong et al., 1998; Schneitler et al., 1998). SN is generated in

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ABBREVIATIONS: SN, secretoneurin; SgII, secretogranin II; CgA, chromogranin A; PACAP, pituitary adenylate cyclase-activating polypeptide; PAC1-R, pituitary adenylate cyclase-activating polypeptide-preferring receptor; VIP, vasoactive intestinal polypeptide; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; PKA, protein kinase A; AP-1, activator protein-1; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; AM, acetoxymethyl ester; RIA, radioimmunoassay; ERK, extracellular signal-regulated kinase; EMSA, electrophoretic mobility shift assay; VOCCs, voltage-operated calcium channels; ATF, activating transcription factors; CRE, cAMP response element; CREB, cAMP response element binding protein.

neuronal and endocrine cells by proteolytic processing at dibasic sites of secretogranin II (SgII), a member of the chromogranin protein family (Fischer-Colbrie et al., 1995). Chromogranins, such as SgII or chromogranin A (CgA), are widely used as markers of the regulated pathway in endocrine cells and of neoplasms derived from these cells (Rosa and Gerdes, 1994), but their function is not completely understood. Recently, cloning of the cDNAs encoding SgII or CgA in phylogenetically distant species has revealed the high conservation of discrete regions within these proteins, which are delimited by potential dibasic cleavage sites (Anouar et al., 1996; Turquier et al., 1999), implying that chromogranins may give rise to such peptides as SN, the sequences of which are preserved during evolution, and which may represent authentic neuropeptides.

Chromogranins are major constituents of the adrenal medullary chromaffin cells, where they are stored in secretory vesicles and released along with catecholamines and neuropeptides upon preganglionic cholinergic stimulation (Fischer-Colbrie et al., 1995). In adrenochromaffin cells, SgII has been shown to be processed to SN (Kirchmair et al., 1993) and other peptides (Anouar et al., 1998) that can be released upon nicotinic stimulation (Wolkersdorfer et al., 1996), probably to participate in the adaptive responses of the adrenal gland during physical or psychological stress.

In addition to acetylcholine, the pleiotropic neuropeptide PACAP (Vaudry et al., 2000) has emerged recently as a potent regulator of chromaffin cell activity after release from the splanchnic nerve fibers innervating the adrenal medulla (Dun et al., 1996). PACAP, acting through the PACAP-preferring receptor, PAC1-R (Tanaka et al., 1998), stimulates the secretion of catecholamines (Przywara et al., 1996; Taupenot et al., 1998) and the expression of the genes encoding catecholamine biosynthetic enzymes (Choi et al., 1999). PACAP also regulates the expression of the neuropeptides enkephalin (Hahm et al., 1998) and vasoactive intestinal polypeptide (VIP; Lee et al., 1999), which are coreleased with catecholamines in vivo upon splanchnic nerve stimulation of adrenochromaffin cells. Although it has been reported that PACAP stimulates CgA gene transcription in PC12 pheochromocytoma cells (Taupenot et al., 1998), its effects on the expression of chromogranin genes and the secretion of their processing products in primary chromaffin cells have not yet been investigated. Such a study may help to elucidate the various signaling pathways and transcription factors that could be activated by PACAP to regulate the transcription of a peptide gene and the secretion of that peptide in postmitotic, primary neuroendocrine cells. In addition, determining the factors that regulate the synthesis of chromogranins and the release of derived peptides in vivo may contribute to the understanding of the function of these neuroendocrine proteins.

We have examined the effect of PACAP on SgII and CgA gene expression, and SN release in cultured bovine adrenal medullary chromaffin cells. The present data show that PACAP increases SN release through multiple pathways, whereas its effect on SgII mRNA expression is mainly mediated by calcium influx and mitogen-activated protein kinase (MAPK) cascades. The effect of PACAP on SgII gene expression is mechanistically more related to the effect of the PKC activator, TPA, than to the effect of the PKA activator, forskolin, or cell-depolarization in chromaffin cells. Finally, the data suggest that PACAP enhances the binding of constitutive AP-1-like transcription factors to the CRE to stimulate SgII gene transcription in chromaffin cells.

Materials and Methods

Reagents. Forskolin, TPA, cycloheximide, nimodipine, chelerythrine, BAPTA-AM and poly-L-lysine were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). H7 was purchased from ICN Pharmaceuticals (Orsay, France) and H89 from Alexis Biochemicals (San Diego, CA). U0126 was obtained from Promega Corp. (Charbonnières, France) and SB 203580 from Calbiochem (Meudon, France).

Peptides. Bovine SN and PACAP38 were synthesized by solid-phase methods, as described previously (Yon et al., 1993; Oulyadi et al., 1997). The peptides were purified by high-performance liquid chromatography and their identity was confirmed by mass spectrometry.

Cell Culture and Treatments. Primary cultures of bovine adrenochromaffin cells were obtained after retrograde perfusion of bovine adrenal glands with 0.1% collagenase (Serlabo, Bonneuil-sur-Marne, France) and 30 U/ml DNase I (Sigma-Aldrich), followed by dissociation of the digested adrenal medullae. The cells were cultured in DMEM (Sigma-Aldrich) supplemented with 5% fetal calf serum (Biowhittaker Europe, Verviers, Belgium) and 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml Fungizone (Life Technologies, Cergy-Pontoise, France). Chromaffin cells were purified by differential plating to remove adherent nonchromaffin cells, as described previously (Anouar et al., 1994). They were plated at a density of 10^6 cells/ml in poly-L-lysine-coated 24-well plates. Chromaffin cells were treated overnight with PACAP or other secretagogues in the presence or absence of protein kinase inhibitors, a protein synthesis inhibitor, or calcium mobilization inhibitors. The inhibitors were dissolved in dimethyl sulfoxide (H7, H89, chelerythrine, BAPTA-AM, nimodipine, SB203580, and U0126) or in the medium (cycloheximide) and were added 30 min before the onset of secretagogue treatment. Forskolin and TPA were dissolved in ethanol. The final concentrations of dimethyl sulfoxide or ethanol never exceeded 0.1% except for BAPTA-AM (0.2%), and the control cells contained the same amount of solvent.

SN Radioimmunoassay (RIA). Antibodies against bovine SN (code number 5010705) were raised by injecting New Zealand rabbits with synthetic bovine SN conjugated to bovine serum albumin and emulsified with Freund's adjuvant. The peptide was iodinated by the chloramine-T method and separated from free iodine on Sep-Pak C18 cartridges using a gradient of acetonitrile (20–52%) in water-0.1% trifluoroacetic acid. The radioiodinated peptide eluted at 40% acetonitrile.

The assay was performed in veronal buffer (0.2 M; pH 8.6) supplemented with 0.4% bovine serum albumin. The antiserum, used at a final dilution of 1:60,000, was incubated with 7,000 cpm of tracer/tube for 48 h at 4°C. Separation of antibody-bound from free peptide was achieved by adding 200 μl of goat anti-rabbit γ-globulins (1:40) and 200 μl of normal rabbit serum (1:150). The incubation was continued for another 2 days. After centrifugation, the supernatant was removed and the pellet was counted on a gamma counter (LKB-Wallac, Rockville, MD). The standard curves were generated using synthetic bovine SN at concentrations ranging from 2.5 to 5,000 pg/tube. The specificity of the assay was ascertained by using unrelated peptides including EM66 and PACAP38. At concentrations up to 10,000 pg/tube, these peptides did not interfere in the SN assay. Statistical analysis was performed using the Student's t test.

Messenger RNA Measurements. RNA was harvested from individual cell culture wells by adding 500 μl of Tri-Reagent (Sigma-Aldrich) according to the manufacturer's instructions. RNA was electrophoresed on denaturing agarose gels, blotted on Hybond NX nylon membranes (Amersham Pharmacia Biotech, Les Ulis, France) and

fixed by UV irradiation. The filters were hybridized with a 773-bp fragment of bovine SgII cDNA, corresponding to nucleotides 781 to 1554 of the original clone described by Fischer-Colbrie et al. (1990), which was labeled by random priming or with an antisense 48-mer oligonucleotide, 5′-TTCTGCAGCATCCTTGGAGGACACCTCT-TCTGTCACCTCTTTCGGCTC-3′, corresponding to nucleotides 488–535 of the bovine CgA cDNA (GenBank accession number X04298), which was labeled by terminal deoxynucleotidyl transferase (Promega) in the presence of [α-^{32}P]dCTP (Amersham Pharmacia Biotech). After autoradiography, the mRNA signals were quantified using a BIO-500 image analysis system (Biocom, Les Ulis, France) and were corrected for RNA loading variations by scanning the ethidium bromide-stained 18S ribosomal RNA of each sample using the DensyLab 2.0.5 software (Bioprobe Systems, Montreuil, France). Statistical analysis was performed using the Student's t test.

Western Blot Analysis. Chromaffin cells were plated in six-well plates (2×10^6 cells in 2 ml of medium in each well) and were treated with 50 nM PACAP for 5, 10, or 30 min before harvesting. Protein extracts were obtained by lysis of the cells with 10 mM Tris-HCl, pH 7.4, 0.05% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride followed by sonication (3×10 s) of the lysate. After centrifugation (12,000g, 15 min), the supernatant was precipitated with 10% trichloroacetic acid, the protein precipitate was washed by ethanol/ether (v/v) and dissolved in electrophoresis-denaturing buffer. Twenty micrograms of protein, quantified by the Bradford protein assay (Bio-Rad, Evry-sur-Seine, France), were analyzed by SDS-polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose membranes (Stratagene, La Jolla, CA). The activation of the p42/44 extracellular-regulated kinases 1/2 (ERK1/2) was examined by using the rabbit polyclonal antibodies Anti-ACTIVE MAPK pAb (Promega) at a 1:5,000 dilution. The activation of the p38 MAPK was examined by using the mouse monoclonal antibody p-p38 (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:200 dilution. The total amount of the enzymes (active and non active) was determined by the Anti-ERK 1/2 pAb (Promega) and p38 (N-20) (Santa Cruz Biotechnology) polyclonal antibodies at 1:5,000 and 1:500 dilutions, respectively. The enzyme-antibody complexes were visualized with the use of an enhanced chemiluminescence Western blotting analysis system (ECL; Amersham Pharmacia Biotech).

Electrophoretic Mobility Shift Assay (EMSA). Chromaffin cells were treated with different secretagogues for 1 h and nuclear extracts were prepared as described previously (Anouar et al., 1994). A double-stranded oligonucleotide containing the SgII CRE region 5′-TCGACCCGGTGACGTCAGTGTG-3′ was used in gel-shifting studies and was labeled by fill-in using [α-^{32}P]dCTP and the Klenow enzyme. Chromaffin cell nuclear extracts (2 μg of protein) were assayed for their binding to the SgII CRE as described (Anouar et al., 1994). In the supershift EMSA, specific antibodies were included as described previously (Anouar et al., 1999). The antibodies used (1 μl/reaction) were affinity-purified IgG fractions recognizing ATF1 p35, CREB-1 p43, and CREM-1 in the case of the anti-ATF1 antibody, all known Fos homologs in the case of the anti-Fos antibody, and all known Jun homologs in the case of anti-Jun antibody. These antibodies were obtained from Santa Cruz Biotechnology and their specificity has been checked previously (Anouar et al., 1999).

Results

PACAP Stimulates SgII, but not CgA, Gene Expression and SN Release in Chromaffin Cells. To study the effect of PACAP38 on chromogranin gene expression, primary cultures of highly purified bovine chromaffin cells were incubated overnight with graded doses of the neuropeptide, and SgII or CgA mRNA levels were measured by Northern blot analysis (Fig. 1, A and B). PACAP induced a dose-dependent stimulation of SgII mRNA levels (up to ~60-fold) with a half-maximal effect at a concentration of 3.39 nM (Fig. 1A). In contrast, CgA mRNA levels were unaffected by PACAP treatment (Fig. 1B), indicating that PACAP exerts a gene-specific effect on members of the chromogranin family in bovine chromaffin cells. To examine whether the stimulatory effect of PACAP on SgII mRNA levels is correlated to an effect of PACAP on the release of the SgII-derived peptide SN, the amount of SN was measured in the medium of chromaffin cells after an overnight PACAP treatment. The concentration of SN was increased in the medium of PACAP-treated cells (up to 4-fold) compared with untreated cells (Fig. 1C). The half-maximal effect was obtained at a concentration of 2.98 nM PACAP. Thus, PACAP stimulates SN release that is accompanied by an increase in SgII gene transcription probably to replenish and maintain the SN stores.

Time-Course of the Effect of PACAP on SgII Gene Expression and SN Release. Treatment of chromaffin cells with PACAP provoked a relatively rapid increase in SgII mRNA levels, which reached 3-fold above control levels after 1 h (Fig. 2A). The stimulatory effect of PACAP gradually increased, reaching ~30-fold after 16 h, and persisted even after 72 h (Fig. 2A). Kinetic experiments also showed that PACAP evoked a ~2-fold increase in SN concentration in the medium within 5 min of treatment (Fig. 2B). SN release remained at approximately the same level during the first hour of treatment, reached a level 4- to 5-fold higher after 16 h, and markedly diminished after 72 h (Fig. 2B). These kinetics are consistent with a rapid and long-lasting effect of PACAP on chromaffin cell activity. PACAP induces an immediate effect on SN release followed by a robust effect on SgII gene expression, enabling a sustained secretory response.

The Effect of PACAP on SgII Gene Expression and SN Release Depends on Calcium Influx. PACAP acting through its type I receptors, has been shown to activate different transduction pathways in chromaffin cells (Tanaka et al., 1998). In particular, PACAP stimulates calcium influx from the external milieu and calcium release from intracellular stores in these cells (Tanaka et al., 1996). To demonstrate the involvement and to determine the source of the second messenger calcium in PACAP-induced SN release and gene transcription, we used either the cytosolic calcium chelator BAPTA-AM (50 μM) or a nonselective calcium channel blocker, the divalent cation nickel (3 mM $NiCl_2$). Both drugs antagonized to a similar extent (40–50% inhibition) the effect of PACAP38 on SgII mRNA levels (Fig. 3A) and SN release (Fig. 3B) in bovine chromaffin cells. The equal efficacy of BAPTA-AM and $NiCl_2$ to reduce the stimulatory action of PACAP on both SN release and SgII gene expression suggests that extracellular calcium plays a major role in these processes. However, nimodipine (10 μM), an antagonist of L-type calcium channels, suppressed SN release (Fig. 3B) without affecting SgII gene expression (Fig. 3A), indicating that PACAP activates various voltage-operated calcium channels (VOCCs) to regulate SN secretion and SgII gene transcription in chromaffin cells.

Differential Involvement of Protein Kinases in the Effect of PACAP on SgII Gene Expression and SN Release. The nonselective protein kinase inhibitor H7 (100 μM) markedly reduced the effect of PACAP on both mRNA levels and SN release (Fig. 4), indicating the importance of protein kinases in the effect of PACAP. Indeed, the effect of PACAP

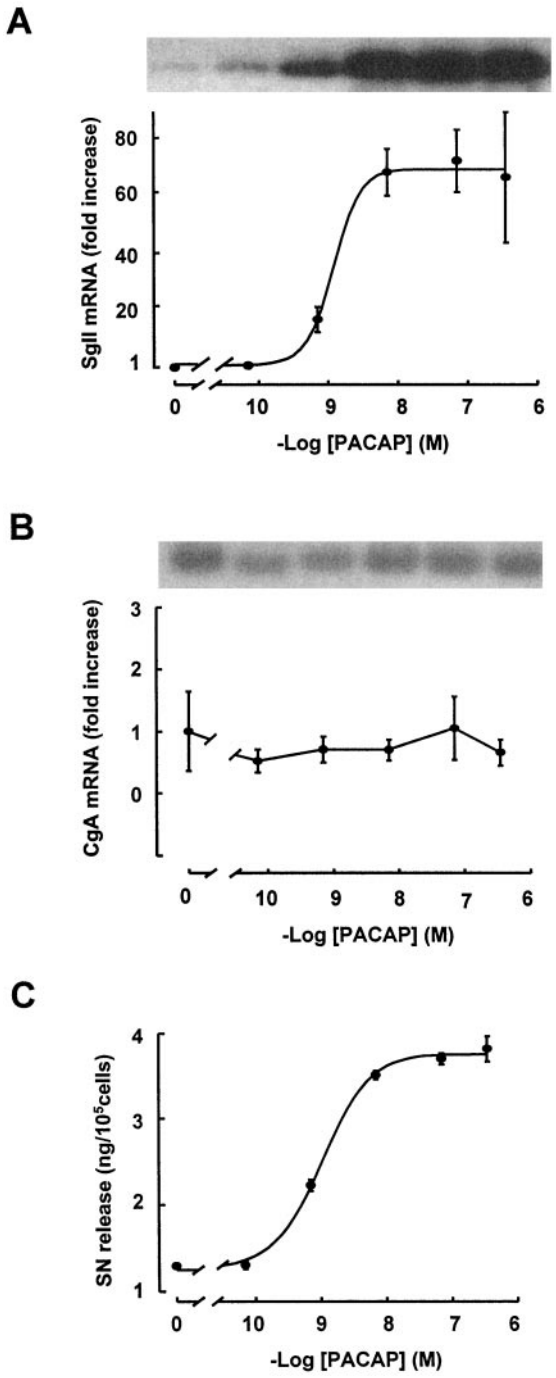

Fig. 1. Effect of PACAP on SgII and CgA mRNA levels, and SN release. A, chromaffin cells were treated overnight with PACAP at concentrations ranging from 7×10^{-11} to 3.5×10^{-7} M and harvested for total RNA and Northern blotting as described under *Materials and Methods*. The blot was hybridized with a bovine SgII cDNA probe. B, the same blot was stripped and hybridized with a ^{32}P-labeled oligonucleotide deduced from bovine CgA cDNA. Messenger RNA signals were quantified by densitometry and corrected for RNA loading variations, which were determined by scanning ethidium bromide-stained 18S RNA. Values are the mean ± S.E.M. of four separate determinations from one experiment and are expressed as a fold increase over control untreated cells. Representative autoradiographic signals obtained with the different PACAP concentrations are shown above each graph. C, media from control or PACAP-treated chromaffin cells were collected and SN-immunoreactivity was assessed by RIA. Values are expressed as nanograms of SN secreted by 10^5 cells and represent the means ± S.E.M. of four separate determinations from one experiment. A second experiment was performed which made it possible to calculate mean EC_{50} values of 3.39 ± 2.20 nM for stimulation of SgII mRNA levels and 2.98 ± 1.95 nM for SN release.

on SN release was also reduced significantly by the PKA inhibitor H89 (20 μM) and by the PKC inhibitor chelerythrine (5 μM) (Fig. 4B). However, the PACAP-induced stimulation of SgII mRNA levels was not affected by the PKA and PKC inhibitors (Fig. 4A), suggesting that the activation of these enzymes by PACAP in chromaffin cells mediates only the increase in SN release, whereas protein kinases other than PKA and PKC may be involved in PACAP-induced SgII gene expression.

Mitogen-Activated Protein Kinases Play a Pivotal Role in PACAP Regulation of SgII Gene Expression in Chromaffin Cells. The p42/44 ERK 1/2 MAPK inhibitor U0126 (10 μM) provoked a 60% decrease in the effect of PACAP on SgII gene expression, whereas the p38 MAPK inhibitor SB203580 (10 μM) did not impair the stimulatory effect of PACAP (Fig. 5A). In contrast, both the ERK1/2 and p38 MAPK inhibitors reduced the effect of PACAP on SN

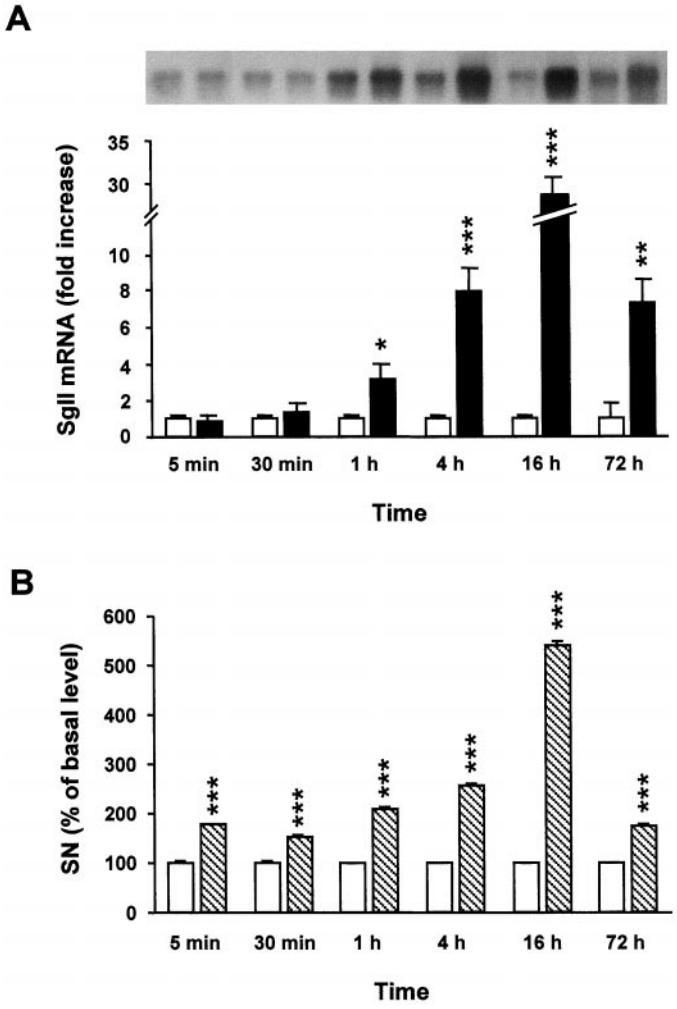

Fig. 2. Time course of PACAP-induced SgII mRNA levels and SN release. Chromaffin cells were incubated in the absence (control; open bars) or presence of 50 nM PACAP for various duration. A, means ± S.E.M. of SgII mRNA levels, expressed as fold increase over corresponding control values, were determined from six determinations for each time point (see legend to Fig. 1). Representative autoradiograms obtained at the different times are shown above the graph. B, means ± S.E.M. of SN concentration in the culture medium, expressed as percentages of basal level (corresponding controls), were determined from six determinations for each time point. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus the corresponding control (Student's t test).

release, although the ERK inhibitor seemed to be more efficacious than the p38 inhibitor (Fig. 5B). Because these results indicated that PACAP would activate both the ERK MAPK- and the p38 MAPK-signaling pathways, the effect of PACAP on ERK1/2 and p38 phosphorylation was examined. Western blot analysis using antibodies directed against ERK1/2 phosphorylated on threonine-183/tyrosine-185, showed that ERK1/2 phosphorylation was strongly increased after 5 min of incubation of chromaffin cells with PACAP and was back to normal after 10 min of treatment (Fig. 6A). Using an antibody recognizing p38 phosphorylated on tyrosine-182, only a slight increase in the phosphorylation of different isoforms of p38 MAPK was observed after 5 min of exposure to PACAP (Fig. 6B). The total level of ERK1/2 (Fig. 6C) or p38 MAPK was not modified by PACAP treatment (Fig. 6D).

The Effects of PACAP and TPA on SgII Gene Expression Are Cycloheximide-Insensitive, whereas the Effects of Forskolin and K^+-Depolarization Are Cycloheximide-Sensitive. To identify the factors required for PACAP action, we assessed the effect of the protein synthesis inhibitor cycloheximide on either PACAP- or protein kinases and calcium influx stimulator-induced SgII gene expression. Incubation of chromaffin cells with PACAP (50 nM), forskolin (25 μM), TPA (100 nM), or high K^+ (25 mM) in the absence or

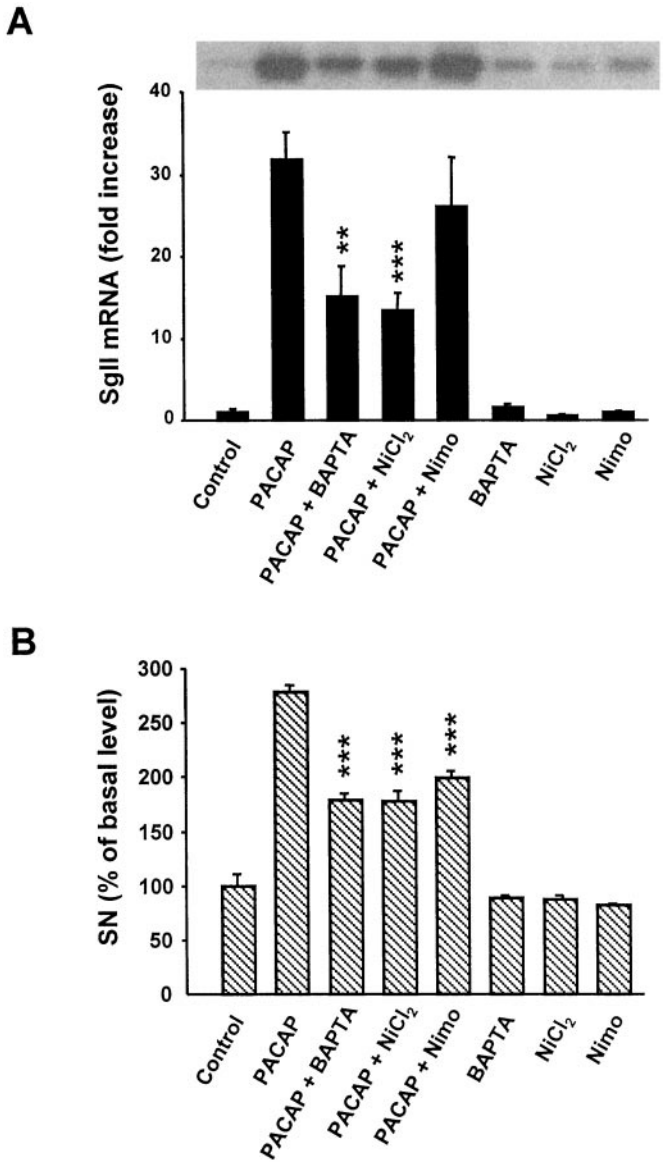

Fig. 3. Effect of calcium mobilization inhibitors on PACAP-induced SgII mRNA levels and SN release. Chromaffin cells were incubated overnight in control conditions or with 50 nM PACAP, in the absence or presence of 50 μM BAPTA-AM, 3 mM $NiCl_2$, or 10 μM nimodipine (Nimo) as described under *Materials and Methods*. A, means ± S.E.M. of SgII mRNA levels, expressed as a fold increase of control values, were determined from three to four independent experiments, each comprising four samples per condition. Variations of SgII mRNA levels were determined as described in the legend to Fig. 1. Representative autoradiographic signals obtained with the different treatments are shown above the graph. B, means ± S.E.M. of SN in the culture medium, expressed as percentages of basal level (Control), were obtained from two to four independent experiments, each comprising four samples per condition. $^{**}p < 0.01$, $^{***}p < 0.001$ versus PACAP alone (Student's t test).

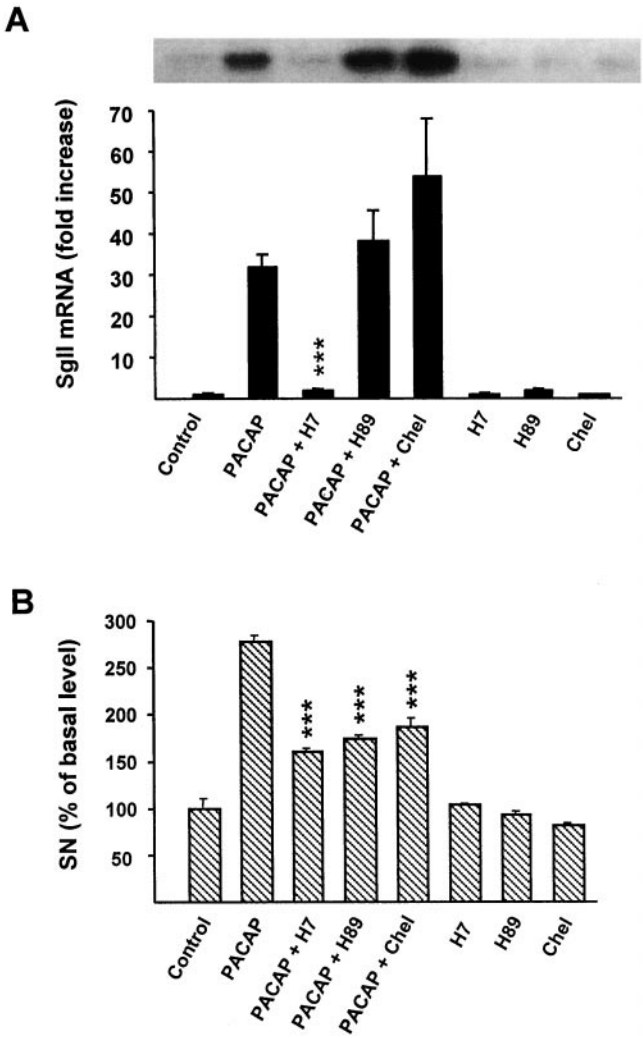

Fig. 4. Effect of protein kinase inhibitors on PACAP-induced SgII mRNA levels and SN release. Chromaffin cells were incubated overnight in control conditions or with 50 nM PACAP, in the absence or presence of 100 μM H7, 20 μM H89, or 5 μM chelerythrine (Chel) as described under *Materials and Methods*. A, means ± S.E.M. of SgII mRNA levels, expressed as a fold increase of control values, were determined from two to four independent experiments, each comprising four samples per condition. Variations of SgII mRNA levels were determined as described in the legend to Fig. 1. Representative autoradiographic signals obtained with the different treatments are shown above the graph. B, means ± S.E.M. of SN in the culture medium, expressed as percentages of basal level (Control), were obtained from two to four independent experiments, each comprising four samples per condition. $^{***}p < 0.001$ versus PACAP alone (Student's t test).

presence of cycloheximide (0.5 μg/ml) revealed that the effect of PACAP, like the effect of TPA, on SgII mRNA levels was not affected by cycloheximide treatment (Fig. 7). In contrast, cycloheximide markedly reduced the effects of forskolin and K^+-induced depolarization on SgII gene expression (Fig. 7). This indicates that the chromaffin cell factors involved in PACAP as well as TPA induction of SgII mRNA levels are pre-existing proteins, whereas the effects of forskolin and potassium require newly synthesized factors to stimulate SgII gene expression.

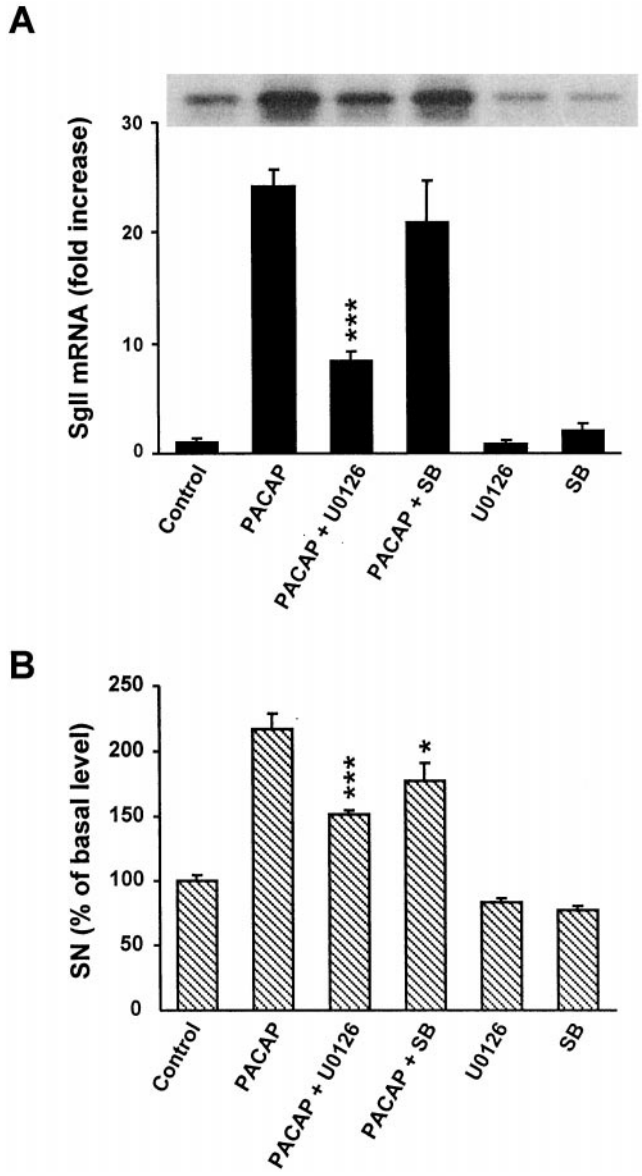

Fig. 5. Effect of MAPK inhibitors on PACAP-induced SgII mRNA levels and SN release. Chromaffin cells were incubated overnight in control conditions or with 50 nM PACAP, in the absence or presence of 10 μM U0126 or 10 μM SB203580, as described under *Materials and Methods*. A, means ± S.E.M. of SgII mRNA levels, expressed as a fold increase of control values, were determined from two to three independent experiments, each comprising four samples per condition. Variations of SgII mRNA levels were determined as described in the legend to Fig. 1. Representative autoradiographic signals obtained with the different treatments are shown above the graph. B, means ± S.E.M. of SN in the culture medium, expressed as percentages of basal level (Control), were obtained from two to three independent experiments, each comprising four samples per condition. $^*p < 0.05$, $^{***}p < 0.001$ versus PACAP alone (Student's t test).

PACAP Increases the Binding Activity of the SgII CRE to AP-1-Like Transcription Factors in Chromaffin Cells. The SgII gene promoter encompasses a consensus CRE that is completely conserved in mammalian species. This element plays a crucial role in basal as well as second messenger-stimulated transcription of the SgII gene in several cell types (Desmoucelles et al., 1999; Mahata et al., 1999). Using gel shifting assay, we have analyzed the SgII CRE binding activity to proteins present in nuclear extracts of chromaffin cells that had been treated by PACAP, forskolin, TPA, or high K^+. PACAP, TPA, and K^+ increased the amount of transcription factors bound to the SgII CRE, whereas forskolin treatment did not alter the CRE binding activity (Fig. 8). To identify the transcription factors that interact with the SgII CRE in chromaffin cells and that may mediate the effect of PACAP on SgII gene transcription, we have added in the CRE binding assays antibodies recognizing members of AP-1 complexes (all members of Fos and all members of Jun families of transcription factors) and CREB/ATF families of transcription factors (Fig. 9). In control conditions, the Fos antibody interfered with most of the binding to the CRE from chromaffin cell nuclear extracts. Both the Jun and the CREB/ATF antibodies partially supershifted the CRE-bound complexes, although the CREB/ATF antibody seemed more efficient (Fig. 9A). Under PACAP stimulation, the increased binding of chromaffin cell transcription factors to the CRE was also mostly supershifted by the anti-Fos, followed by the anti-CREB/ATF and the anti-Jun antibodies (Fig. 9B). Because Fos homodimers cannot interact with DNA, these results indicate that the CRE binds preferentially to AP-1-like protein complexes containing a Fos family member combined to a member of the CREB/ATF or Jun families. When nuclear extracts from forskolin-, TPA- or K^+-stimulated chromaffin cells were used, the efficiency of

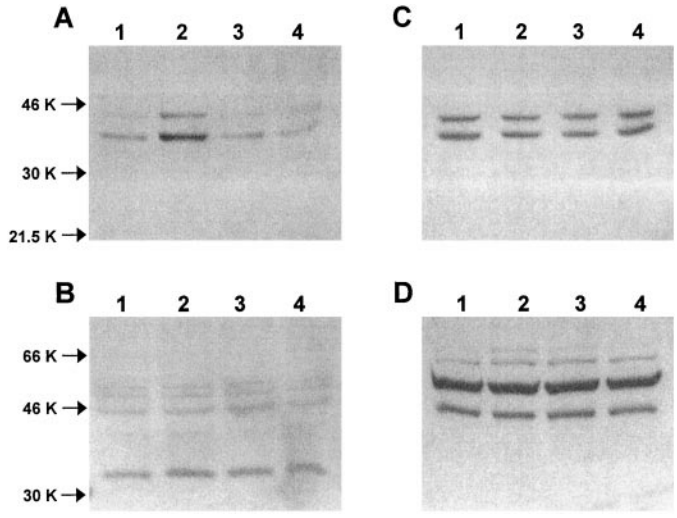

Fig. 6. Effect of PACAP on the phosphorylation of ERK1/2 and p38 MAPK. Chromaffin cells were incubated in control conditions for 30 min (lane 1) or with 50 nM PACAP for 5 min (lane 2), 10 min (lane 3), or 30 min (lane 4), and protein extracts were analyzed by Western blotting as described under *Materials and Methods*. A, phosphorylation of ERK1/2 was probed with the Anti-ACTIVE MAPK pAb. B, phosphorylation of p38 was probed with the antibody p-p38. Note that for p38, four isoforms named α, β, δ, and γ with molecular masses around 44 kDa have been described (Hale et al., 1999). C, the total amount of ERK1/2 was determined by using the Anti-ERK 1/2 pAb. D, the total amount of p38 was assessed by using the p38 (N20) antibody. Molecular mass markers (in kiloDaltons) are indicated.

the antibodies in supershifting CRE-bound proteins was quite similar to that observed under PACAP stimulation, except that the Jun antibody supershifted more efficiently the protein complexes present in the TPA-stimulated extracts compared with the other extracts (Fig. 9, C–E).

Discussion

The present study has shown that PACAP38 stimulates SgII gene expression and SN release and has described the transduction pathways involved in the effect of PACAP on bovine adrenochromaffin cells in primary culture.

Chromaffin cells of the adrenal medulla are neuroendocrine, nondividing cells that express and secrete a wide variety of neuropeptides in addition to catecholamines, making primary cultures of these cells a very useful model-system to study neuropeptide regulation as it could occur in vivo. Recently, it has been shown that PACAP evokes a long-lasting effect on chromaffin cell secretion that persists for several hours (Babinski et al., 1996) as opposed to the acute effect of nicotinic cholinergic stimulation on the release of chromaffin granule content, which desensitizes rapidly (Boksa and Livett, 1984).

Among its various effects on chromaffin cell activity, PACAP induced SgII gene expression and SN release without affecting the expression of the gene encoding the other chromogranin, CgA. It is interesting to note that the latter is under the control of PACAP in PC12 pheochromocytoma cells (Taupenot et al., 1998). It has also been shown that nicotinic (Wolkersdorfer et al., 1996) as well as histaminergic (Bauer et al., 1993) stimulation of chromaffin cells leads to an increase in SgII but not CgA biosynthesis. From these data, it appears that the SgII gene is highly regulated in chromaffin cells, as is also the case for various neuropeptide genes, such as the proenkephalin gene (Eiden et al., 1984; Farin et al., 1990; Hahm et al., 1998), the galanin gene (Anouar et al., 1999), and the VIP gene (Eiden et al., 1983; Lee et al., 1999), which are also regulated by PACAP, nicotine, or histamine. The induction of SgII mRNA by PACAP is accompanied by an increase in SN release from chromaffin cells consistent with the concept of stimulus-secretion-biosynthesis coupling in chromaffin cells, according to which the cells synthesize secretory products to replenish their stores after stimulation (MacArthur and Eiden, 1996). However, the various molecular mechanisms involved in the regulation of gene transcription and peptide secretion in chromaffin cells under physiological *trans*-synaptic stimuli are not completely elucidated.

The stimulatory effect of PACAP on SN release occurred within a few minutes and lasted up to 72 h, although attenuation of the response was observed after 3 days of treatment. These data confirm and extend those reported previously by Babinski et al. (1996) showing that PACAP exerts long-lasting effects (several hours) on the secretory activity of chromaffin cells. In addition to this immediate stimulation of SN secretion, PACAP significantly increased SgII mRNA

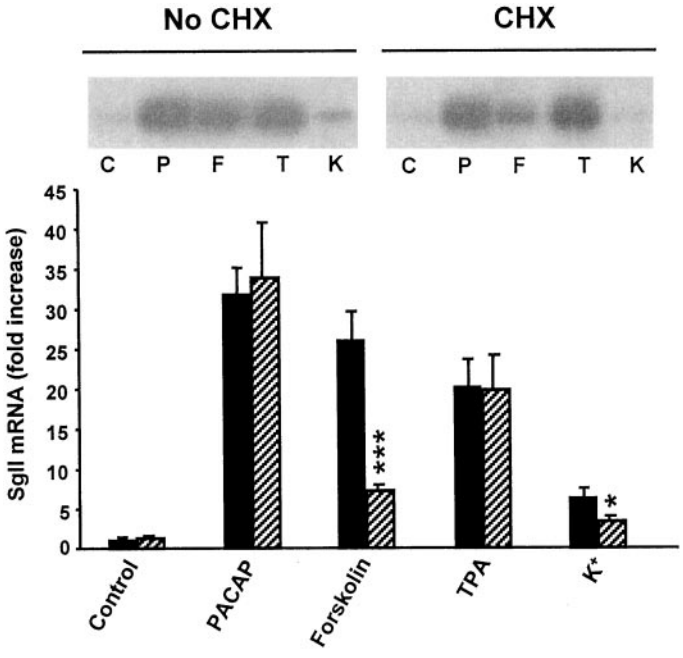

Fig. 7. Effect of protein synthesis inhibition on SgII gene expression stimulated by PACAP, forskolin, TPA, or high K^+. Chromaffin cells were incubated overnight in control conditions or with 50 nM PACAP, 25 μM forskolin, 100 nM TPA, or 25 mM K^+ in the absence (black columns) or presence (hatched columns) of 0.5 μg/ml cycloheximide (CHX). Cells were harvested for mRNA analysis as described under *Materials and Methods*. Data are from three to four independent experiments, each comprising four to six samples per condition and are presented as mean values ± S.E.M. Representative autoradiographic signals obtained with the different treatments are shown above the graph. $^*p < 0.05$, $^{***}p < 0.001$ for cells treated with cycloheximide compared with cells not treated with cycloheximide (Student's t test).

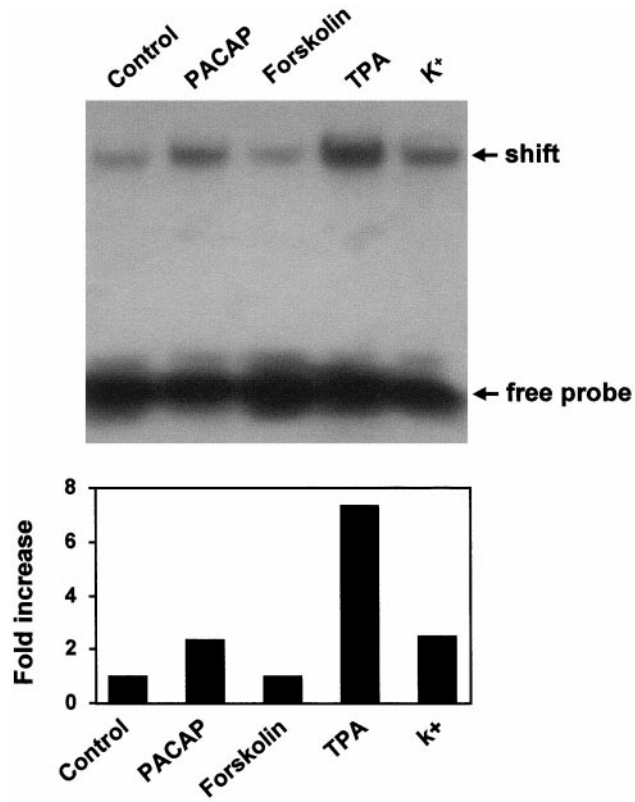

Fig. 8. Effect of PACAP, forskolin, TPA, or high K^+ on the binding activity of the SgII CRE to chromaffin cell nuclear extracts. Chromaffin cells were incubated for 1 h in control conditions or with 50 nM PACAP, 25 μM forskolin, 100 nM TPA, or 25 mM KCl (K^+) and harvested for nuclear extracts. EMSA was carried out with 2 μg of proteins and 40,000 cpm of ^{32}P-labeled SgII CRE oligonucleotide probe as described under *Materials and Methods*, and the reactions were analyzed on 0.25× Tris-Borate-EDTA (TBE)-polyacrylamide gel. The arrows indicate the shifted and free probe.

levels (3-fold) within 1 h of exposure, indicating that PACAP also exerts a rapid effect on SgII gene expression in chromaffin cells. These results suggest that PACAP acts at the transcriptional level, at least in part, to up-regulate SgII mRNA levels. The fact that SgII mRNA concentration remained elevated for 72 h indicates that long-term secretion of SN is supported by a long-lasting increase in gene expression in chromaffin cells.

The type I PACAP receptor, which is found in bovine adrenal medullary cells, is known to be coupled with several second messengers including cAMP, IP_3, and calcium (Spengler et al., 1993). Consistently, PACAP induces cAMP and IP_3 as well as calcium mobilization in bovine chromaffin cells (Tanaka et al., 1996). Pharmacological stimulation by forskolin, TPA, or K^+ depolarization has suggested that different second messengers regulate SgII biosynthesis in chromaffin cells (Fischer-Colbrie et al., 1990), indicating that PACAP may recruit multiple signaling pathways to regulate SgII gene transcription and SN release. The present study revealed that calcium influx is a key process in the regulatory mechanisms underlying the effect of PACAP, because calcium channel blockers strongly inhibited both SN secretion and SgII gene transcription. In addition, nimodipine blocked the PACAP-induced SN release, a result that is in good agreement with the fact that PACAP induces the opening of L-type VOCCs in chromaffin cells (Tanaka et al., 1996). However, we found that nimodipine does not affect PACAP-induced SgII gene expression, suggesting that non L-type VOCCs, such as P-, Q- or N-types, which are also present on bovine chromaffin cells (Artalejo et al., 1994), may be activated by PACAP to ultimately up-regulate the expression of the SgII gene. Alternatively, mechanisms involving store-

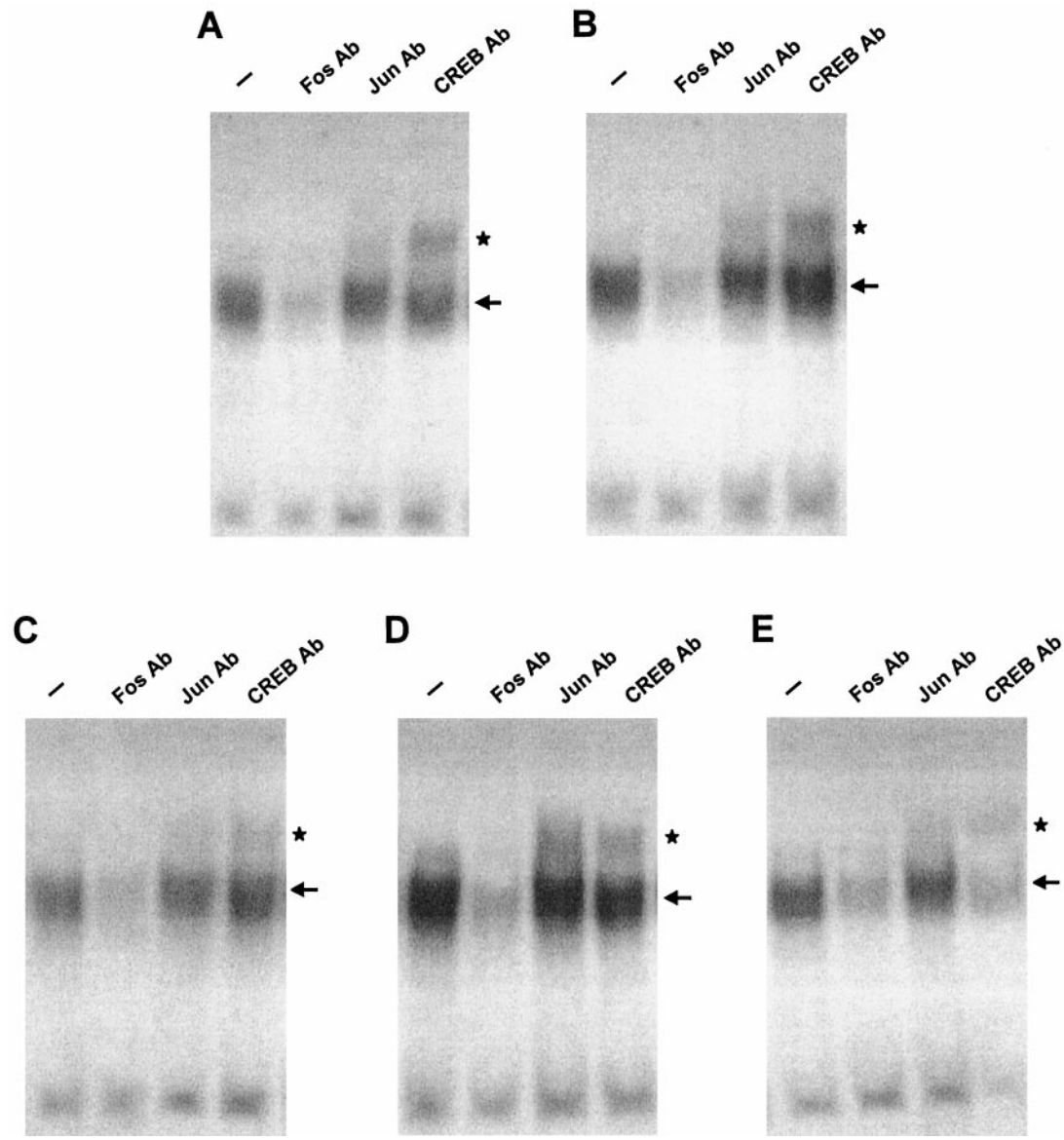

Fig. 9. Supershift EMSA of the SgII CRE by Fos, Jun, and CREB/ATF antibodies. Effects of PACAP, forskolin, TPA, or high K^+. A, supershift EMSA with nuclear extracts from control chromaffin cells using antibodies against Fos family members (Fos Ab), Jun family members (Jun Ab), and ATF1/CREB/CREM transcription factors (CREB Ab). B, supershift EMSA with nuclear extracts from PACAP-treated chromaffin cells as described in A. C, supershift EMSA with nuclear extracts from forskolin-treated chromaffin cells as described in A. D, supershift EMSA with nuclear extracts from TPA-treated chromaffin cells as described in A. E, supershift EMSA with nuclear extracts from K^+-treated chromaffin cells as described in A. The asterisks denote the supershifted probe and the arrows indicate the shifted probe.

operated calcium channels whose activity is associated with low-magnitude calcium influx, such as those described for the long-term effect of PACAP on catecholamine secretion (Taupenot et al., 1999) could be implicated in SgII gene expression upon PACAP stimulation. It has been shown that the stimulatory effect of PACAP on the VIP gene is also dependent on calcium influx (Lee et al., 1999), whereas the regulation of the genes encoding proenkephalin (Hahm et al., 1998) and chromogranin A (Taupenot et al., 1998) by PACAP in chromaffin cells is calcium-independent. Altogether, these observations indicate that several Ca^{2+} routes could be activated by PACAP to differentially regulate the secretion and biosynthesis of the various components of chromaffin cell granules.

PKA and PKC inhibitors also markedly attenuated PACAP-induced stimulation of SN secretion by chromaffin cells but they did not affect SgII mRNA levels. Because PACAP-induced SgII gene expression was significantly reduced by H7, a general protein kinase inhibitor, the involvement of other protein kinases such as MAPK has been considered. PACAP has been shown to activate the MAP kinases ERK1/2 in PC12 cells (Barrie et al., 1997) but no data are available in primary chromaffin cells. In fact, we found that the mitogen-activated protein kinase kinase inhibitor U0126 was very effective in reducing the stimulatory activity of PACAP on SgII gene expression. Concurrently, the p38 MAPK seems to be involved only in the secretory process of SN and only to a limited extent. In agreement with these observations, PACAP induced a marked increase in ERK1/2 phosphorylation and a modest effect, if any, on p38 phosphorylation in chromaffin cells. We cannot rule out the possibility that the minor decrease observed with the p38 inhibitor could be caused not only by an effect on PACAP-induced SN secretion, but perhaps also by a slight effect on SgII processing to secretoneurin. This is the first study to show an effect of PACAP on MAPK, particularly ERK1/2, in primary cultures of chromaffin cells and their major role in gene transcription, at least for the SgII gene and its derived secretory product SN.

It appears from these data that PACAP activates several pathways in bovine chromaffin cells to regulate the release of a peptide such as SN and its gene transcription, as summarized in Fig. 10. According to this model, PACAP stimulation of chromaffin cells most probably via PAC1-R, provokes the secretion of SN through activation of multiple pathways including calcium influx, PKA, PKC, ERK1/2, and to a lesser extent p38 MAPK. Concurrently, PACAP induction of SgII gene expression seems to recruit only calcium entry through a nonL-type VOCC, and ERK1/2 (Fig. 10). Based on these results, it can be hypothesized that, since secretion is a rapid event for the cell response as shown by the time course experiments, numerous signal transduction cascades are concomitantly activated by PACAP to trigger SN release,

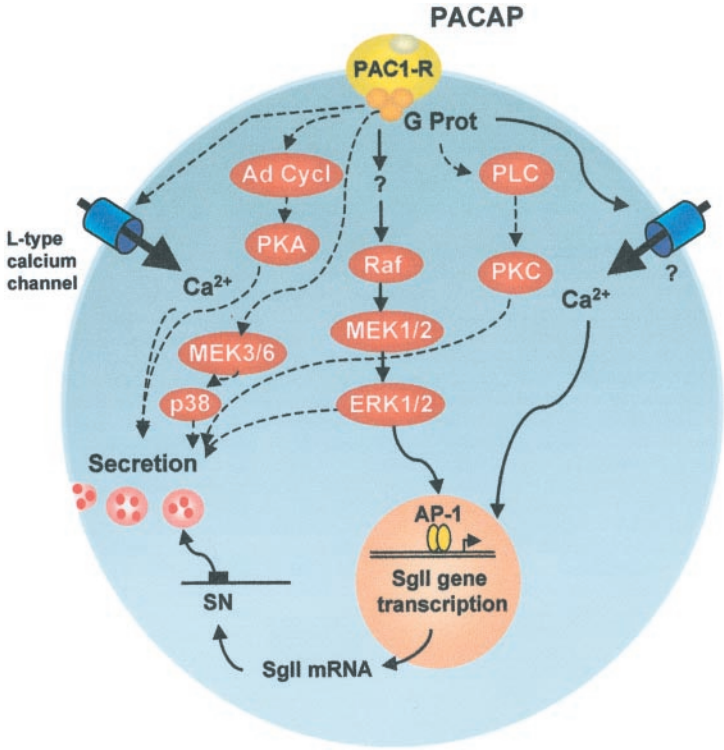

Fig. 10. Model of the transduction pathways involved in the effect of PACAP on SN release and SgII gene transcription in chromaffin cells. The results reported herein suggest that PACAP binding to its receptor (PAC-1R) on chromaffin cells provokes calcium influx through L-type calcium channels and serine/threonine protein kinase (PKA, PKC, and ERK 1/2) activation, probably by phosphorylation, leading to secretion of SN. Note that the p38 MAPK pathway plays only a minor role in SN secretion compared with calcium or the other protein kinases, the inhibition of which reduced by about 50% the PACAP-evoked SN release. PACAP also stimulates extracellular calcium entry through a non-L-type calcium channel and triggers a transient increase in ERK 1/2 phosphorylation leading to induction of SgII mRNA levels, which occurs within 1 h and lasts for several hours. The signaling pathways implicated in the effect of PACAP most probably impinge on the binding activity of CRE to AP-1-like transcription factors, leading to enhanced SgII gene transcription. The dashed arrows indicate the activated transduction pathways leading to SN secretion and the solid line arrows indicate the pathways involved in SgII gene expression under PACAP stimulation. Although these mechanisms may also play a role in the acute effects of PACAP, the present model only refers to the transduction pathways recruited during long-term exposure to PACAP. Ad cycl, adenylyl cyclase; G prot, G protein; PAC1-R, type I PACAP receptor; PLC, phospholipase C.

whereas activation of specific transduction pathways is necessary and sufficient to evoke SgII gene transcription which will ultimately replenish the releasable pool of SN. In this respect, it is noteworthy that PACAP provoked a transient increase of ERK1/2 phosphorylation which apparently returned to baseline within 10 min after treatment. The induction of SgII mRNA levels was observed as early as 1 h after the onset of PACAP exposure, and lasted at least for 16 h. Altogether, these data suggest that PACAP triggers the phosphorylation of ERK1/2 which rapidly leads to an increase in SgII gene transcription. The fact that SgII mRNA levels still increased after 16 h suggests either that the initial induction of ERK1/2 activity is sufficient to trigger a long-lasting stimulation of SgII mRNA levels or that a low level of ERK1/2 phosphorylation is maintained during all the period of SgII gene expression evoked by PACAP.

At this time, the molecular basis of the coupling between PAC1-R and MAP kinases or calcium influx in bovine chromaffin cells is not known (Fig. 10) except that PKA and PKC are not implicated since inhibitors of these kinases do not affect PACAP-induced SgII mRNA elevation. Cooperation between calcium influx and MAPK to regulate gene transcription has been well evidenced by several studies, notably in PC12 cells, showing a stimulus-dependent induction of MAP kinases through calcium influx and subsequent activation of the protooncogene small G-protein Ras or stimulation of PKA or PKC (Rosen et al., 1994; Tang et al., 1998; Sakai et al., 1999). Further studies are required to elucidate the mechanisms by which PACAP induces MAPK phosphorylation and activation in primary chromaffin cells.

It is now well established that the routes leading to stimulation of neuropeptide gene expression by second messengers culminate either in the induction of immediate-early genes which can be blocked by protein synthesis inhibitors or in the activation by phosphorylation/dephosphorylation of pre-existing transcription factors which is unaffected by protein synthesis blockers (Bacher et al., 1996; MacArthur and Eiden, 1996; Anouar et al., 1999). These transcriptional trans-activators usually belong to the CREB/ATF and Fos/Jun families of basic-leucine zipper proteins which interact with *cis*-acting elements known as CRE (cAMP and calcium response element)- or TRE (TPA response element)-like sequences on neuropeptide gene promoters (Bacher et al., 1996; MacArthur and Eiden, 1996; Eiden et al., 1998; Anouar et al., 1999). Prototypical transduction mechanisms that have been well characterized in several systems include the cAMP/PKA/CREB which can be induced by the adenylate cyclase-stimulating agent forskolin or by intracellular calcium increase, or the diacyl glycerol/PKC/AP-1 which can be activated by TPA.

Although the activation of the PKA and PKC pathways by forskolin and TPA, respectively, stimulates SgII gene transcription, activation of these pathways by PACAP does not seem to impinge on SgII gene transcription in chromaffin cells. It is thus conceivable that, under PACAP stimulation, the ERK1/2 pathway, but not the PKA or PKC pathways, plays a predominant role in SgII gene transcription. In addition, the present study shows that the factors involved in the effect of PACAP on SgII gene expression differ from those implicated in the effect of forskolin, since the former was insensitive to protein synthesis inhibition by cycloheximide while the latter was cycloheximide-sensitive. In this respect, the effect of PACAP on SgII gene transcription is similar to the effect of TPA which is also mediated by pre-existing trans-activating factors. Furthermore, we found that both PACAP and TPA increased the binding activity of the SgII CRE to proteins of chromaffin cell nuclear extracts, while forskolin did not exert such an effect, indicating some mechanistic relationship between PACAP and TPA in regulating SgII gene transcription in chromaffin cells. Thus, these secretagogues probably affect the activity of constitutive transcription factors by a post-translational modification leading to increased binding to the SgII CRE. The nature of the CRE-bound proteins has been studied by using antibodies to Fos, Jun and ATF/CREB. This analysis revealed that AP-1-like complexes composed of a Fos-like protein associated with a Jun-like or an ATF/CREB-like protein interact with the SgII CRE, and that PACAP or TPA increased the binding of these pre-existing AP-1-like transcription factors to the SgII CRE. According to the model shown in Fig. 10, PACAP stimulates SgII gene expression by activating calcium influx and MAP kinases that in turn stimulate the activity of AP-1-like transcription factors. These modified AP-1-related transcription factors bind more efficiently to the CRE, resulting in enhanced transcription of the SgII gene in chromaffin cells. In support of this scheme, it is now well known that MAP kinases regulate AP-1 activity in a variety of tissues (Karin, 1995).

In conclusion, this study has revealed that PACAP stimulates SN release and SgII gene transcription in primary cultures of bovine chromaffin cells and has identified the complex potential signaling pathways underlying this regulation. Some aspects of the mechanisms described such as the activation of MAP kinases or the involvement of AP-1-like transcription factors may be of general relevance for neuropeptide gene regulation by the neurotrophic factor PACAP in neuroendocrine cells. Finally, the tight regulation of SN release and gene transcription, akin to the regulation of neuropeptides, by *trans*-synaptic stimuli including PACAP in chromaffin cells supports the view that SN can be regarded as an authentic neuropeptide.

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References

Agneter E, Sitte HH, Stockl-Hiesleitner S, Fischer-Colbrie R, Winkler H and Singer EA (1995) Sustained dopamine release induced by secretoneurin in the striatum of the rat: a microdialysis study. *J Neurochem* **65:**622–625.

Anouar Y, Desmoucelles C, Yon L, Leprince J, Breault L, Gallo-Payet N and Vaudry H (1998) Identification of a novel secretogranin II-derived peptide (SgII(187- 252)) in adult and fetal human adrenal glands using antibodies raised against the human recombinant peptide. *J Clin Endocrinol Metab* **83:**2944–2951.

Anouar Y, Jegou S, Alexandre D, Lihrmann I, Conlon JM and Vaudry H (1996) Molecular cloning of frog secretogranin II reveals the occurrence of several highly conserved potential regulatory peptides. *FEBS Lett* **394:**295–299.

Anouar Y, Lee HW and Eiden LE (1999) Both inducible and constitutive activator protein-1-like transcription factors are used for transcriptional activation of the galanin gene by different first and second messenger pathways. *Mol Pharmacol* **56:**162–169.

Anouar Y, MacArthur L, Cohen J, Iacangelo AL and Eiden LE (1994) Identification of a TPA-responsive element mediating preferential transactivation of the galanin gene promoter in chromaffin cells. *J Biol Chem* **269:**6823–6831.

Artalejo CR, Adams ME and Fox AP (1994) Three types of Ca^{2+} channel trigger secretion with different efficacies in chromaffin cells. *Nature* **367:**72–76.

Babinski K, Bodart V, Roy M, De Lean A and Ong H (1996) Pituitary adenylate-cyclase activating polypeptide (PACAP) evokes long-lasting secretion and de novo biosynthesis of bovine adrenal medullary neuropeptides. *Neuropeptides* **30:**572–582.

Bacher B, Wang X, Schulz S and Hollt V (1996) Induction of proenkephalin gene expression in cultured bovine chromaffin cells is dependent on protein synthesis of AP-1 proteins. *J Neurochem* **66:**2264–2271.

Barrie AP, Clohessy AM, Buensuceso CS, Rogers MV and Allen JM (1997) Pituitary adenylyl cyclase-activating peptide stimulates extracellular signal-regulated kinase 1 or 2 (ERK1/2) activity in a Ras-independent, mitogen-activated protein kinase/ERK kinase 1 or 2-dependent manner in PC12 cells. *J Biol Chem* **272:** 19666–19671.

Bauer JW, Kirchmair R, Egger C and Fischer-Colbrie R (1993) Histamine induces a gene-specific synthesis regulation of secretogranin II but not of chromogranin A and B in chromaffin cells in a calcium-dependent manner. *J Biol Chem* **268:**1586–1589.

Boksa P and Livett BG (1984) Desensitization to nicotinic cholinergic agonists and K^+, agents that stimulate catecholamine secretion, in isolated adrenal chromaffin cells. *J Neurochem* **42:**607–617.

Choi HJ, Park SY and Hwang O (1999) Differential involvement of PKA and PKC in regulation of catecholamine enzyme genes by PACAP. *Peptides* **20:**817–822.

Desmoucelles C, Vaudry H, Eiden LE and Anouar Y (1999) Synergistic action of upstream elements and a promoter-proximal CRE is required for neuroendocrine cell-specific expression and second-messenger regulation of the gene encoding the human secretory protein secretogranin II. *Mol Cell Endocrinol* **157:**55–66.

Dun NJ, Tang H, Dun SL, Huang R, Dun EC and Wakade AR (1996) Pituitary adenylate cyclase activating polypeptide-immunoreactive sensory neurons innervate rat adrenal medulla. *Brain Res* **716:**11–21.

Dunzendorfer S, Schratzberger P, Reinisch N, Kahler CM and Wiedermann CJ (1998) Secretoneurin, a novel neuropeptide, is a potent chemoattractant for human eosinophils. *Blood* **91:**1527–1532.

Eiden LE, Anouar Y, Hsu CM, MacArthur L and Hahm SH (1998) Transcription regulation coupled to calcium and protein kinase signaling systems through TRE- and CRE-like sequences in neuropeptide genes. *Adv Pharmacol* **42:**264–268.

Eiden LE, Eskay RL, Scott J, Pollard H and Hotchkiss AJ (1983) Primary cultures of bovine chromaffin cells synthesize and secrete vasoactive intestinal polypeptide (VIP). *Life Sci* **33:**687–693.

Eiden LE, Giraud P, Dave JR, Hotchkiss AJ and Affolter HU (1984) Nicotinic receptor stimulation activates enkephalin release and biosynthesis in adrenal chromaffin cells. *Nature* **312:**661–663.

Farin CJ, Kley N and Hollt V (1990) Mechanisms involved in the transcriptional activation of proenkephalin gene expression in bovine chromaffin cells. *J Biol Chem* **265:**19116–19121.

Fischer-Colbrie R, Gutierrez J, Hsu CM, Iacangelo A and Eiden LE (1990) Sequence analysis, tissue distribution and regulation by cell depolarization, and second messengers of bovine secretogranin II (chromogranin C) mRNA. *J Biol Chem* **265:**9208–9213.

Fischer-Colbrie R, Laslop A and Kirchmair R (1995) Secretogranin II: molecular properties, regulation of biosynthesis and processing to the neuropeptide secretoneurin. *Prog Neurobiol* **46:**49–70.

Hahm SH, Hsu CM and Eiden LE (1998) PACAP activates calcium influx-dependent and -independent pathways to couple met-enkephalin secretion and biosynthesis in chromaffin cells. *J Mol Neurosci* **11:**43–56.

Hale KK, Trollinger D, Rihanek M and Manthey CL (1999) Differential expression and activation of p38 mitogen-activated protein kinase alpha, beta, gamma, and delta in inflammatory cell lineages. *J Immunol* **162:**4246–4252.

Karin M (1995) The regulation of AP-1 activity by mitogen-activated protein kinases. *J Biol Chem* **270:**16483–16486.

Kirchmair R, Hogue-Angeletti R, Gutierrez J, Fischer-Colbrie R and Winkler H (1993) Secretoneurin-a neuropeptide generated in brain, adrenal medulla and other endocrine tissues by proteolytic processing of secretogranin II (chromogranin C). *Neuroscience* **53:**359–365.

Kong C, Gill BM, Rahimpour R, Xu L, Feldman RD, Xiao Q, McDonald TJ, Taupenot L, Mahata SK, Singh B, et al (1998) Secretoneurin and chemoattractant receptor interactions. *J Neuroimmunol* **88:**91–98.

Lee HW, Hahm SH, Hsu CM and Eiden LE (1999) Pituitary adenylate cyclase-activating polypeptide regulation of vasoactive intestinal polypeptide transcription requires Ca^{2+} influx and activation of the serine/threonine phosphatase calcineurin. *J Neurochem* **73:**1769–1772.

MacArthur L and Eiden L (1996) Neuropeptide genes: targets of activity-dependent signal transduction. *Peptides* **17:**721–728.

Mahata SK, Mahata M, Livsey CV, Gerdes HH, Huttner WB and O'Connor DT (1999) Neuroendocrine cell type-specific and inducible expression of the secretogranin II gene: crucial role of cyclic adenosine monophosphate and serum response elements. *Endocrinology* **140:**739–749.

Oulyadi H, Davoust D and Vaudry H (1997) A determination of the solution conformation of secretoneurin, a neuropeptide originating from the processing of secretogranin II, by 1H-NMR and restrained molecular dynamics. *Eur J Biochem* **246:**665–673.

Przywara DA, Guo X, Angelilli ML, Wakade TD and Wakade AR (1996) A noncholinergic 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.

Rosa P and Gerdes HH (1994) The granin protein family: markers for neuroendocrine cells and tools for the diagnosis of neuroendocrine tumors. *J Endocrinol Invest* **17:**207–225.

Rosen LB, Ginty DD, Weber MJ and Greenberg ME (1994) Membrane depolarization and calcium influx stimulate MEK and MAP kinase via activation of Ras. *Neuron* **12:**1207–1221.

Sakai T, Furuyama T, Ohoka Y, Miyazaki N, Fujioka S, Sugimoto H, Amasaki M, Hattori S, Matsuya T and Inagaki S (1999) Mouse semaphorin H induces PC12 cell neurite outgrowth activating Ras- mitogen-activated protein kinase signaling pathway via Ca^{2+} influx. *J Biol Chem* **274:**29666–29671.

Schneitler C, Kahler C, Wiedermann CJ, Hogue-Angeletti R and Fischer-Colbrie R (1998) Specific binding of a ^{125}I-secretoneurin analogue to a human monocytic cell line. *J Neuroimmunol* **86:**87–91.

Spengler D, Waeber C, Pantaloni C, Holsboer F, Bockaert J, Seeburg PH and Journot L (1993) Differential signal transduction by five splice variants of the PACAP receptor. *Nature* **365:**170–175.

Tanaka K, Shibuya I, Nagamoto T, Yamashita H and Kanno T (1996) Pituitary adenylate cyclase-activating polypeptide causes rapid Ca2+ 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.

Tanaka K, Shibuya I, Uezono Y, Ueta Y, Toyohira Y, Yanagihara N, Izumi F, Kanno T and Yamashita H (1998) Pituitary adenylate cyclase-activating polypeptide causes Ca^{2+} release from ryanodine/caffeine stores through a novel pathway independent of both inositol trisphosphates and cyclic AMP in bovine adrenal medullary cells. *J Neurochem* **70:**1652–1661.

Tang K, Wu H, Mahata SK and O'Connor DT (1998) A crucial role for the mitogen-activated protein kinase pathway in nicotinic cholinergic signaling to secretory protein transcription in pheochromocytoma cells. *Mol Pharmacol* **54:**59–69.

Taupenot L, Mahata M, Mahata SK and O'Connor DT (1999) Time-dependent effects of the neuropeptide PACAP on catecholamine secretion : stimulation and desensitization. *Hypertension* **34:**1152–1162.

Taupenot L, Mahata SK, Wu H and O'Connor DT (1998) Peptidergic activation of transcription and secretion in chromaffin cells. Cis and trans signaling determinants of pituitary adenylyl cyclase-activating polypeptide (PACAP). *J Clin Invest* **101:**863–876.

Turquier V, Vaudry H, Jegou S and Anouar Y (1999) Frog chromogranin A messenger ribonucleic acid encodes three highly conserved peptides. Coordinate regulation of proopiomelanocortin and chromogranin A gene expression in the pars intermedia of the pituitary during background color adaptation. *Endocrinology* **140:**4104–4112.

Vaudry H and Conlon JM (1991) Identification of a peptide arising from the specific post-translation processing of secretogranin II. *FEBS Lett* **284:**31–33.

Vaudry D, Gonzalez BJ, Basille M, Yon L, Fournier A and Vaudry H (2000) Pituitary adenylate cyclase-activating polypeptide and its receptors from structure to functions. *Parmacol Rev* **52:**269–324.

Wolkersdorfer M, Egger C, Laslop A and Fischer-Colbrie R (1996) Nicotine and prostaglandin E induce secretogranin II levels in bovine chromaffin cells. *Mol Brain Res* **38:**260–266.

Yon L, Feuilloley M, Chartrel N, Arimura A, Fournier A and Vaudry H (1993) Localization, characterization and activity of pituitary adenylate cyclase-activating polypeptide in the frog adrenal gland. *J Endocrinol* **139:**183–194.

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