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Pituitary Adenylate Cyclase Activating Polypeptide (PACAP) Regulates Expression of Catecholamine Biosynthetic Enzyme Genes in Bovine Adrenal Chromaffin Cells

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Abstract

Pituitary adenylate cyclase activating polypeptide (PACAP) elevates levels of the mRNAs encoding the catecholamine synthesizing enzymes tyrosine hydroxylase (TH), dopamine β-hydroxylase (DBH), and phenylethanolamine *N*-methyltransferase (PNMT) in primary cultures of bovine adrenal chromaffin cells. PACAP potently (in nanomolar concentrations) increases the amount of mRNA for each of the three catecholamine biosynthetic enzymes. At 10 n*M* PACAP, TH and DBH mRNA levels increase approx 10-fold; 1 n*M* PACAP produces an approx 2.5-fold elevation of PNMT mRNA. In contrast to depolarizing or cholinergic stimuli, PACAP does not enhance expression of 5' upstream regions of the PNMT gene transiently transfected into chromaffin cells. Nor does PACAP stimulate the rate of PNMT gene transcription, thereby indicating that the effects of this neuropeptide do not involve enhanced transcription of this gene. However, after 16 h in the presence of transcriptional inhibitors, more PNMT mRNA is present in cultures treated with PACAP relative to control cultures, whereas amounts of TH and DBH mRNAs are not changed. PACAP likely elevates PNMT mRNA levels posttranscriptionally, possibly by stabilizing this message against degradation. Thus, although PACAP is an effective regulator for expression of all three catecholamine enzyme genes, its mechanism of action on PNMT mRNA appears to be distinctive from its effects on TH and DBH gene transcription.

Index Entries: Tyrosine hydroxylase; dopamine β-hydroxylase; phenylethanolamine *N*-methyl transferase; adrenal medulla; transcriptional regulation.

Introduction

In 1989, Arimura and colleagues (Miyata et al., 1989) isolated from ovine hypothalamus a novel neuropeptide, pituitary adenylate cyclase activating polypeptide (PACAP), which stimulates adenylate cyclase in pituitary cells and peripheral neural tissues. Two equally potent forms, PACAP-38 and PACAP-27 (possessing the N-terminal 27 residues of PACAP-38) are alternatively processed

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from a precursor protein of 176 amino acids (Kimura et al., 1990; Miyata et al., 1990; Arimura, 1992). PACAP is a member of the secretin/glucagon family, sharing amino acid sequence identity of 68% with vasoactive intestinal polypeptide (VIP) and 37% with secretin (Ogi et al., 1990; Arimura, 1992).

PACAP functions as a neuromodulator (transmitter) in the central and peripheral nervous system. Its stimulatory actions are mediated through at least two different receptors (Types I and II) (Arimura et al., 1991; Shivers et al., 1991; Deutsch and Sun, 1992; Hashimoto et al., 1993; Masuo et al., 1993; Spengler et al., 1993), which in turn are translated from multiple splice variants (rev. in Spengler et al., 1993). The type I receptor, which is highly selective for PACAP (relative to VIP), is present in the adrenal gland (Arimura et al., 1991; Shivers et al., 1991; Hashimoto et al., 1993; Masuo et al., 1993) and positively coupled to G_s and phospholipase C (Spengler et al., 1993).

PACAP immunoreactivity is localized in fibers innervating rat and bovine adrenal medulla (Guo et al., 1994; Marley and McLeod, 1995), and in noradrenergic chromaffin cells of rat adrenal (Shiotani et al., 1995). Moreover, PACAP potently stimulates catecholamine release from isolated perfused rat adrenals (Wakade, 1988; Wakade et al., 1992; Przywara et al., 1995), from sympathetic neurons (May and Braas, 1995), from PC12 pheochromocytoma cells (Watanabe et al., 1990; Strong et al., 1992), and from single rat chromaffin cells (Chowdhury et al., 1994) through a noncholinergic mechanism (Przywara et al., 1995, 1996).

Intracellularly, PACAP elicits concentration-dependent increases in enzymatic activity of tyrosine hydroxylase (TH; tyrosine-3-monooxygenase, EC 1.14.16.2) (Rius et al., 1994), and in TH mRNA in bovine chromaffin cells (Rius et al., 1994), rat PC12 pheochromocytoma cells (Strong et al., 1992), and sympathetic neurons (May and Braas, 1995). Although it has not been explicitly demonstrated, it is likely that PACAP-mediated stimulation of TH gene expression is mediated through a cAMP responsive element (CRE) in 5' regulatory region of the TH gene (Fossom et al., 1991; Kilbourne et al., 1992). A functional CRE has likewise been identified in the 5' portion of the DBH gene (McMahon and Sabban, 1992; Kim et al., 1994). However, the PNMT genes sequenced thus far lack a canonical CRE. The authors sought to resolve whether PACAP influences expression of the dopamine beta-hydroxylase (DBH; 3,4-dihydroxyphenylalanine, ascorbate:oxygen oxidoreductase, EC 1.14.17.1), and phenylethanolamine *N*-methyltransferase (PNMT; EC 2.1.1.28) genes in primary cultures of bovine adrenal chromaffin cells as it does for expression of TH mRNA. Other features distinguish PACAP action on adrenergic and noradrenergic cells of the adrenal medulla. Specifically, PACAP preferentially stimulates release of epinephrine (in a 7:1 ratio relative to norepinephrine [Guo and Wakade, 1994]). Moreover, immunocytochemical detection reveals that PACAP is *not* localized in epinephrine-producing cells of the adrenal medulla (Shiotani et al., 1995).

Because PNMT possesses structural and regulatory aspects distinct from the other catecholamine synthetic enzyme genes, the mechanism by which PACAP elevates levels of PNMT mRNA has been analyzed in detail. The effects of PACAP on both the transcriptional expression and the rates of synthesis and degradation for PNMT mRNA have been examined. In contrast to the influences of neurally mediated depolarizing and cholinergic stimuli (Evinger et al., 1994), PACAP does not alter the rate of PNMT gene transcription. Instead, the authors report that one aspect of PACAP influence on PNMT expression is a stabilization of PNMT mRNA against degradation. Thus, distinctive mechanisms appear to mediate the effects of PACAP on catecholamine enzyme gene expression in noradrenergic and adrenergic cells of the adrenal medulla.

Materials and Methods

Primary Chromaffin Cell Cultures

Primary chromaffin cell cultures were established from fresh bovine adrenal medullae (Max Insel Cohen, Inc.) using the Renografin gradient fractionation method as previously described (Evinger et al., 1994). Cells were plated in Falcon (10 cm, cat. no. 3003) tissue cultures dishes at a density of 2×10^5 cells/cm^2 in Dulbecco's MEM: F12 medium (1:1), 10% fetal calf serum, 10 m*M* HEPES, pH 7.4, and penicillin/streptomycin/neomycin (Gibco-BRL, Gaithersburg, MD). After 10–12 h incubation at 37°C, 7% CO_2, unattached cells were aspirated, washed by centrifugation, and plated in fresh medium onto uncoated 60-mm Falcon culture dishes; this produced a population of >98% chromaffin cells as ascertained by neutral red staining (Ross et al., 1990). Cells were permitted to attach for 24 h prior to treatment with PACAP-38 (Peninsula Laboratories, Belmont, CA) for Northern blot and nuclear run-on transcriptional analyses. For transient transfection analyses, cells were permitted to attach 6–8 h before application of DNA-calcium phosphate precipitates. Cultures were washed 12–13 h later, and incubated with fresh medium for 6 h prior to treatment with PACAP, thereby effecting an equivalent time in culture as utilized for Northern analyses before addition of PACAP to chromaffin cells. After the initial 18–20 h in culture, TH and PNMT mRNA levels in untreated bovine chromaffin cell control cultures did not change substantially for 48–72 h (Carroll et al., 1991, and unpublished observations).

Northern Blot Analysis

For preparation of total RNA, chromaffin cells were harvested by scraping in phosphate-buffered saline (PBS) (Gibco-BRL), then lysed in 50 m*M* Tris, pH 8.0, 100 m*M* NaCl, 5 m*M* $MgCl_2$, 0.5% Nonidet P40, 4 U/mL RNasin with 10 μg/mL glycogen added to improve recovery (Ausubel et al., 1991). RNAs were recovered following extractions by phenol and chloroform, then precipitation with ethanol. They were fractionated on denaturing formaldehyde agarose gels then transferred to Gene Screen Plus (NEN/DuPont) for hybridization with ^{32}P-labeled, random primed cDNA probes. Northern analysis was performed by sequential hybridizations using bovine TH (Carroll et al., 1991), DBH (Hwang and Joh, 1993), and PNMT (Baetge et al., 1986) cDNAs. Following hybridization and autoradiography, relative levels of mRNA were quantified by two-dimensional laser scanning (LKB UltroScan, Turku, Finland) or phosphoimager scanning (ImageQuaNT). Normalization for loading efficiency was achieved by expressing mRNA densitometric signals relative to the signal generated by hybridization with an 18S RNA or GAPDH cDNA probe.

In Vitro Nuclear Run-On Assays

In vitro nuclear run-on assays to measure rates of gene transcription were performed as previously described (Evinger et al., 1992b). Chromaffin cell nuclei (Evinger et al., 1992b, 1994) were isolated from three 60-mm dishes for each treatment following 1 h incubation with PACAP (1 n*M*), KCl (50 m*M*), muscarine (100 μ*M*), or DMEM (control). Equal numbers (1.2×10^6) of washed nuclei from the pooled cultures for each treatment group were added to the transcription mixture containing 0.4 m*M* ATP, 0.4 m*M* CTP, and 250 μCi each of [α^{32}P]UTP and -GTP (NEN, specific activity 600–800 Ci/mmol). After 20 min at 22°C, transcription was terminated by digestion with proteinase K (100 μg/mL) at 37°C for 30 min. Following digestion with DNase and proteinase K, heterogeneous nuclear (hn) RNAs were phenol extracted and ethanol precipitated. After trichloroacetic acid precipitation, total cpm incorporated into hnRNAs were determined by scintillation counting.

^{32}P-labeled hnRNAs were hybridized at 42°C for 72 h with denatured PNMT cDNA and pUC 18 DNAs (Stratagene, La Jolla, CA) (Ausubel et al., 1991) fixed on nitrocellulose filters (Schleicher and Schuell, Dassell, Germany, BA-83). Equivalent cpm (3.3×10^7) of hnRNAs for each treatment were hybridized with duplicate filters containing denatured DNAs (1 μg). After washing twice in 0.3*M* NaCl, 10 m*M* EDTA, 1% SDS, filters were incubated with RNases A and T_1 to digest unhybridized transcripts, then washed extensively in 15 m*M* NaCl, 10 m*M* EDTA, at 60–65°C. The proportion of specifically bound hnRNAs was calculated by subtracting counts bound nonspecifically to pUC DNA from total counts hybridized.

PNMT Promoter Constructs

The following constructs, containing up to 863 bp of sequence 5' to the PNMT transcription start site, were assembled from portions of the 3 kb rat PNMT promoter (designated PNMT 3K-CAT in Ross et al., 1990) by subcloning these fragments into luciferase reporter vectors. (from $^-$863 to $^-$391) pXP_2TK containing 472 bp (from $^-$391 to $^-$863 bp) of the PNMT promoter was constructed by subcloning the *Hin*dIII to *Nhe*I fragment into the *Hin*dIII and *Xba*I sites of the thymidine kinase (TK) promoter (Ross et al., 1990), then ligating this fragment into the pXP_2 luciferase vector (Nordeen, 1988), provided by D. O'Connor, UCSD. The (from $^-$863 to $^+$8) pGL-Basic construct was obtained by ligating the 871 bp *Hin*dIII-*Xho*I fragment digested from PNMT 3K-CAT into the promoterless, enhancerless pGL_2-Basic vector (Promega, Madison, WI). The upstream (from $^-863$ to $^-440$) pGL-Pro construct was prepared by ligating the *Hin*cII-*Kpn*I fragment from PNMT 3K-CAT into the pGL_2-Promoter vector (Promega), containing the heterologous SV40 promoter, via a *Hin*dIII linker at the *Sma*I site. The proximal (from $^-442$ to $^+8$) pGL-Basic construct was achieved by insertion of the *Kpn*I-*Xho*I fragment from PNMT 3K-CAT into pGL_2-Basic.

Transient Transfections

Transient transfections into primary bovine chromaffin cells were performed as detailed in Ross et al. (1990), using the calcium phosphate procedure (Ausubel et al., 1991). At 6–8 h following differential plating, chromaffin cells (5×10^6 cells/60-mm dish) were transfected with 30 μg plasmid DNA plus 10 μg Rous Sarcoma Virus-β-galactosidase (RSV-β-gal) DNA (Edlund et al., 1985) as an internal standard for normalization of transfection and expression efficiencies. Cells were washed with DMEM 12–13 h later, then treated with regulators after 6–8 h. Cells were harvested, washed, and stored at –70°C prior to lysis for measurement of luciferase activity (by modification of Promega protocol) with a Monolight 2010 Luminometer (Analytical Luminescence Laboratory). This assay was linear from 2 pg (corresponding to 100 relative light units, RLU) up to 2 μg using purified beetle luciferase (Analytical Luminescence Labs). The chromogenic substrate o-nitrophenyl galactopyranoside (ONPG) was used to measure RSV-β-gal activity. Luciferase activity (expressed in RLU) is expressed relative to RSV-β-gal activity to correct for sample-to-sample variation in viability of primary chromaffin cell cultures.

Results

Influence of PACAP on TH, DBH, and PNMT mRNA Levels in Chromaffin Cells

The ability of PACAP to influence expression of DBH and PNMT catecholamine synthetic enzyme mRNAs has been compared with that for TH mRNA in bovine chromaffin cells using PACAP concentrations previously shown to elicit maximal catecholamine release. Primary bovine chromaffin cell cultures were treated in triplicate for 16 h with concentrations of PACAP ranging 0.1–10 n*M*, then harvested for isolation of total RNA. Cultures treated with muscarine (100 μ*M*) or KCl (50 m*M*) were included as cholinergic and depolarizing agents previously demonstrated to stimulate transcription of the PNMT gene (Evinger et al., 1994). Sequential Northern blot hybridizations to TH, DBH, and PNMT cDNAs were performed with subsequent normalization to the signal generated by probing with an 18S RNA cDNA. Autoradiographic intensities produced by each catecholamine enzyme cDNA are shown for a representative blot (Fig. 1A) with two-dimensional densitometric scan data for each lane depicted in Fig. 1B. Blots containing RNAs from triplicate cultures were probed by hybridization with the TH ($n = 4$), DBH ($n = 3$), and PNMT ($n = 6$) cDNAs (n represents the number of experiments). Because of the inherent variability in absolute values among individual experiments with primary cultures, data shown are representative of typical experiments, and responses between experiments are expressed as fold change relative to control cultures.

Levels of each of the catecholamine enzyme gene mRNAs increased in response to PACAP treatment, with optimal effects achieved by nanomolar PACAP concentrations for all three mRNAs (Fig. 1). However, differences existed between the effects of PACAP on TH and DBH mRNA expression and its effects on PNMT mRNA. Consistent with previous reports (Strong et al., 1992; Rius et al., 1994), PACAP elevated TH mRNA levels in a dose-dependent manner. In the authors' chromaffin cell cultures, a greater maximal induction (10.1-fold vs control) than reported previously (Rius et al., 1994) was obtained using 10 n*M* PACAP (Fig. 1B). Higher concentrations of PACAP (e.g., 50 and 100 n*M*) did not produce a greater mRNA response (not shown). Similarly, this study establishes for the first time that PACAP induces DBH mRNA optimally at the same concentration (10 n*M*), and to a comparable extent (11.6-fold induction vs control) as that for TH message; higher concentrations of PACAP did not further increase the level of DBH mRNA. In contrast, PNMT mRNA levels were highest at 1 n*M* PACAP; treatment with 5, 10, and 50 n*M* resulted in mean mRNA amounts for two

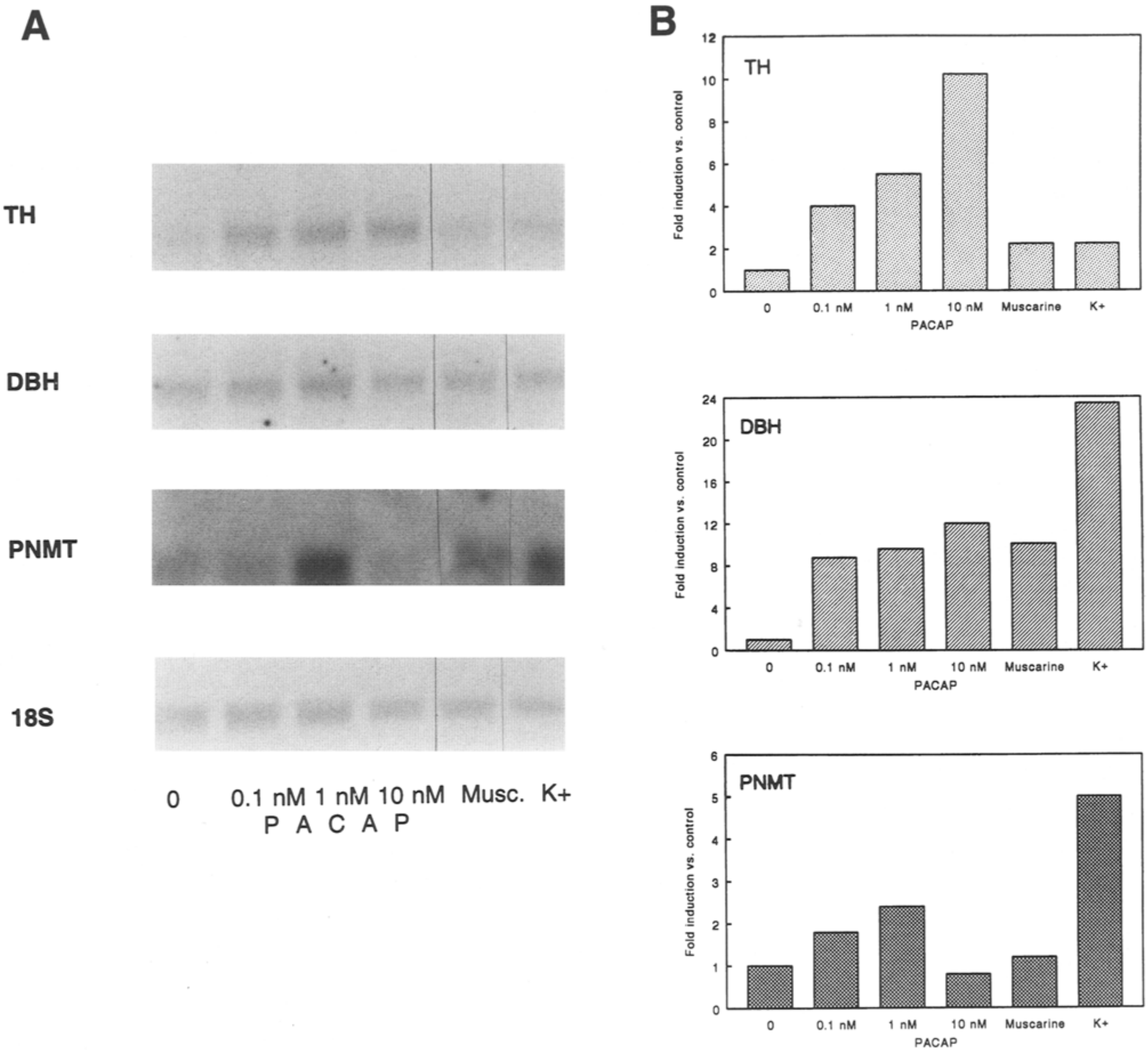

Fig. 1. PACAP elevates steady state levels of TH, DBH and PNMT mRNAs. **(A)** Northern analysis. Bovine chromaffin cell cultures were treated for 16 h with PACAP in increasing concentrations (0, 0.1, 1, and 10 n*M*), muscarine (100 μ*M*), or KCl (50 m*M*). Northern blot analysis was performed on total chromaffin cell RNA (15 μg) prepared from triplicate cultures, first hybridizing with PNMT cDNA, exposing for autoradiography, then stripping and sequentially reprobing with TH, DBH and 18S rRNA cDNAs. **(B)** Quantitation of PNMT, TH, and DBH mRNAs. Densitometric intensities of the autoradiograms in (A) were determined by two-dimensional laser scanning. Intensities for each mRNA are normalized to 18S rRNA and data are expressed as fold induction relative to saline-treated controls following treatment with PACAP (0, 0.1, 1, and 10 n*M*), muscarine (100 μ*M*), and KCl (50 m*M*).

experiments (not shown) that were 58, 46, and 50%, respectively, of cultures treated with 1 n*M* PACAP. Following treatment of primary chromaffin cells with 1 n*M* PACAP, the fold induction for PNMT mRNA ranged 1.4–3.4 with a mean of 2.5 ± 0.3 ($n = 6$ experiments).

PACAP (10 n*M*) produced greater stimulation of TH mRNA production (~10-fold) than did muscarine (100 μ*M*) (twofold) (Fig. 1); its effect on DBH mRNA expression was also significant (~10-fold), but only slightly greater than the five- to sevenfold effect of muscarine on this mRNA. The effect of PACAP was greater than that of K$^+$-mediated (50 m*M*) depolarization (twofold) for TH mRNA, but less than depolarization effects on DBH mRNA (21.2-fold induction vs control). In contrast, the influence of PACAP on PNMT mRNA levels (2.4-fold induction) was less than that evoked by K$^+$-mediated depolarization (Fig. 1B) (Evinger et al., 1994). Although in this specific experiment, the normalized densitometric intensity value for muscarine response was not considerably greater than control, greater muscarine response is typically observed (e.g., a mean of sixfold in Evinger et al., 1994). Notably, PACAP produced increased mRNA levels for all three catecholamine genes in primary chromaffin cells. However, its influence on PNMT mRNA was characterized by a lower concentration necessary to achieve maximal effect, and by a lower magnitude of response relative to that seen for TH and DBH mRNAs.

Effects of PACAP on PNMT Promoter Expression

The ability of forskolin, a stimulator of adenylate cyclase, to elevate PNMT mRNA levels in chromaffin cells (Carroll et al., 1991; Stachowiak et al., 1994) in the absence of a CRE, is suggestive that PACAP influence on PNMT mRNA production may be mediated in a different manner from that postulated for the TH and DBH genes. For this reason, the ability of PACAP to influence transcription of the PNMT gene was assessed by expression of transiently transfected PNMT promoter-reporter constructs in chromaffin cells, and nuclear run-on transcription assays.

The effects of PACAP on expression of the PNMT promoter were examined in transiently transfected bovine adrenal chromaffin cell cultures. Constructs containing 863 bp, the distal 472 bp, the distal 419 bp, and the proximal 435 bp of the upstream 5' PNMT promoter were assembled in luciferase reporter gene vectors (pGL$_2$ or pXP$_2$) as described in Methods and designated (from $^-$863 to $^+$8) pGL-Basic, (from $^-$863 to $^+$391) pXP$_2$ TK, (from $^-$863 to $^+$440) pGL-Pro, and (from $^-$440 to $^+$8) pGL-Basic, respectively (Fig. 2). Chromaffin cells were transfected with PNMT-luciferase constructs, washed, then incubated in duplicate or triplicate 6–8 h later with PACAP (1 n*M*), or KCl (50 m*M*) for an additional 16–18 h. Luciferase activity was assayed in cell extracts, and data normalized to the activity of cotransfected RSV-β-gal. Transfection with each promoter construct and treatment with the indicated regulators were repeated in at least three separate experiments, in duplicate or triplicate, with at least two preparations of supercoiled plasmid DNA. Data were expressed as fold induction relative to control cultures for each construct.

PACAP (1 n*M*) did not produce stimulation of reporter gene activity above that of DMEM treated controls for PNMT promoter constructs (Fig. 2). Although PNMT constructs with heterologous (TK and SV40) promoters showed severalfold higher levels of basal activity than those in pGL-Basic, no significant stimulation of reporter gene activity above untreated controls was seen following PACAP (1 n*M*) or 10 n*M* (not shown) treatment of cells. Furthermore, no change relative to control was observed when cells were transfected with the either full length PNMT promoter (from $^-$863 to $^+$8) (pGL-Basic) or the native PNMT promoter distal regions on heterologous promoters {(from $^-$863 to $^+$391) pXP$_2$ TK and (from $^-$863 to $^+$440) pGL-Pro}. K$^+$-induced depolarization produced 3.5- and 3.6-fold rises relative to controls following transfection with (from $^-$863 to $^+$440) pGL-Pro and (from $^-$863 to $^+$391) TK pXP$_2$, respectively. None of these treatments influenced reporter gene activity for the proximal (from $^-$440 to $^+$8) pGL-Basic construct or the vector alone, consistent with previous reports (Evinger et al., 1992a; Hemmick and Evinger, 1993) that neurally responsive elements are located upstream of the $^-$440 bp *Kpn*I site in the PNMT promoter.

Effects of PACAP on Rate of PNMT Gene Transcription

In vitro nuclear run-on assays were performed to resolve whether PACAP could alter the rate of PNMT gene transcription. Chromaffin cells were treated with PACAP (1 n*M*), K$^+$ (50 m*M*), muscarine (100 μ*M*) or saline for 1 h, the interval previously shown to be optimal for detecting changes

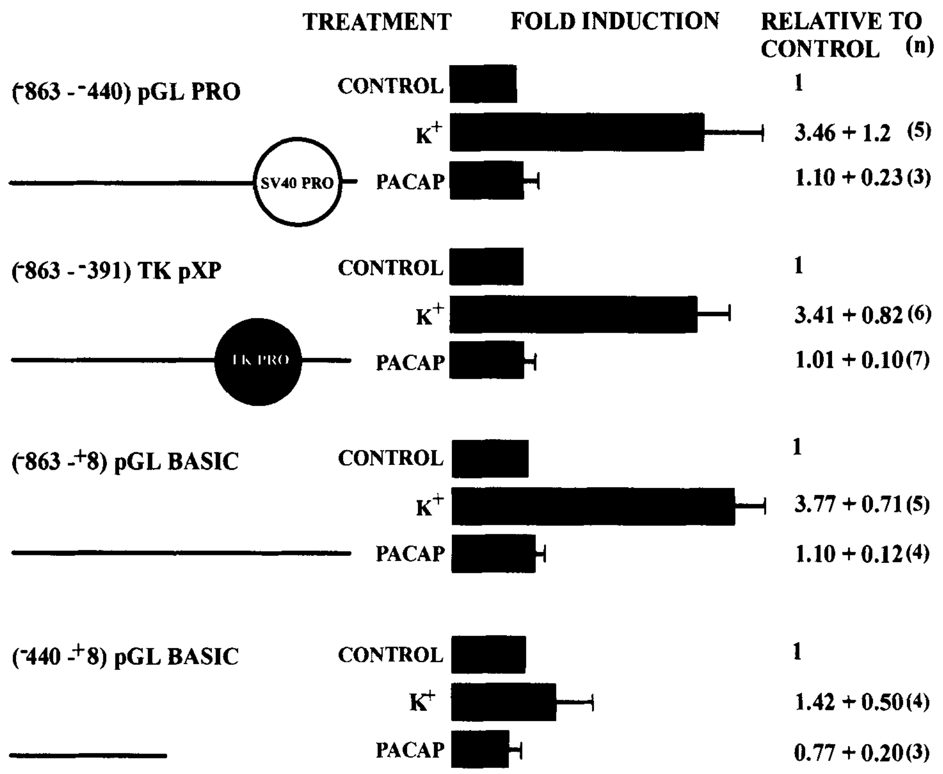

Fig. 2. Effect of PACAP on PNMT promoter expression. Primary bovine adrenal chromaffin cells were transiently transfected with 30 µg luciferase reporter constructs containing 5' upstream regions of the rat PNMT gene plus 10 µg RSV-β-gal (Edlund et al., 1985; Ross et al., 1990). Cultures were treated as detailed in Methods with PACAP (1 n*M*) or KCl (50 m*M*) for 16 h. Expression of the luciferase reporter is depicted relative to that of DMEM-treated controls; in each experiment, data are normalized to the expression of the cotransfected RSV-β-gal construct and are expressed as the mean of duplicate or triplicate determinations for each treatment. The data are expressed as the mean ±SE for the number of experiments designated in parentheses. PNMT constructs were evaluated using both native promoters, (from $^-$863 to $^+$8) pGL-Basic and (from $^-$440 to $^+$8) pGL-Basic, and heterologous promoters, (from $^-$863 to $^-$440) pGL-Pro and (from $^-$863 to $^-$391) pXP$_2$ TK.

in PNMT transcription rate elicited directly by hormonal (Evinger et al., 1992b), neurally mediated (Evinger et al., 1990), and pharmacological (Evinger et al., 1994) agents. Nuclei were isolated, then permitted to elongate transcripts in the presence of radioactive ribonucleotide triphosphates (Evinger et al., 1994). After filter hybridization of the ^{32}P-labeled hnRNAs to denatured plasmid DNAs fixed to nitrocellulose, specific counts hybridized to PNMT cDNA were averaged for duplicate samples and presented for two experiments (Table 1). In Experiment 1, treatment with PACAP did not stimulate PNMT gene transcription (0.9-fold compared to control), whereas K$^+$ depolarization produced a 2.6-fold induction. In Experiment 2, the slight increase in PNMT transcriptional rate after PACAP treatment was not significant when compared to the 7.7-fold stimulation elicited by K$^+$ or the 3.3-fold stimulation produced by muscarine, magnitudes similar to the authors' previous report for these agents (Evinger et al., 1994). The use of a PNMT cRNA antisense probe for hybridization also detected no change: Specifically bound cpm were 1.2-fold relative to the nonspecific RNA control (not shown). Influence on α-tubulin gene transcription was minimal with all treatments in Experiment 2, thereby excluding nonspecific transcriptional stimulation by PACAP, muscarine, or K$^+$. In conjunction with transfection data (Fig. 2), these responses indicated that PACAP did not elevate PNMT mRNA levels by stimulating PNMT gene transcription.

Table 1
Effects of PACAP on Rate of PNMT Transcription

	Total counts hybridized			Specific counts hybridized		Fold induction	
	PNMT	α-Tubulin	pUC	PNMT	α-Tubulin	PNMT	α-Tubulin
Experiment 1							
Control	2072		1446	626		1.0	
K^+	3001		1395	1606		2.6	
PACAP	**1710**		**1130**	**580**		**0.9**	
Experiment 2							
Control	2867	3024	2700	167	324	1.0	1.0
K^+	3864	2684	2401	1283	283	7.7	.87
Muscarine	3818	3729	3275	543	454	3.3	1.4
PACAP	**2754**	**2999**	**2553**	**201**	**446**	**1.2**	**1.3**

Nuclei were prepared from bovine chromaffin cell cultures after treatment for 1 h with DMEM (control), KCl (50 m*M*), muscarine (100 μ*M*), and PACAP (1 n*M*). Nuclear run-on assays were performed as previously described (Evinger et al., 1992) with ^{32}P-labeled hnRNAs isolated for hybridization to 1 μg denatured PNMT, α-tubulin, or pUC 18 DNAs fixed to duplicate nitrocellulose filters. Following digestion with RNases A+T1 and washing in 15 m*M* NaCl, 10 m*M* EDTA, 1% SDS at 65°C for 4 h, filters were counted by liquid scintillation spectroscopy. Specific counts hybridized were determined by subtracting counts nonspecifically hybridized to pUC 18 DNA from total counts hybridized for each cDNA. K^+-mediated depolarization and muscarine produce stimulation (two- to sevenfold) of PNMT gene transcription as reported previously (Evinger et al., 1994). PACAP does not significantly influence the rate of PNMT transcription. Expression of α-tubulin is not influenced by these treatments.

Effect of PACAP on Degradation Rates for PNMT mRNA

To explore the possibility that PACAP might alter the stability of PNMT mRNA, chromaffin cells were treated with two separate inhibitors of transcription. Cultures were first treated with the inhibitor α-amanitin alone (at 1 μg/mL to inhibit ~80% chromaffin cell and >98% PNMT gene transcription, [Evinger and Joh, 1989]) or in combination with PACAP (1 n*M*), then harvested after 16 h, the interval producing maximal effects of PACAP on PNMT mRNA. More PNMT mRNA was present in chromaffin cells treated with PACAP plus α-amanitin (Fig. 3, lane 4) than in cultures treated with α-amanitin alone. This level is greater than that seen in saline treated control cultures (lane 1), cells treated with PACAP alone (lane 2), or cells treated with α-amanitin alone (lane 3). The 1.8-fold greater amount of PNMT mRNA following PACAP treatment at 16 h (lane 2) than that at 0 h (not shown), in this experiment, represents a typical level of induction by PACAP. When analyzed in multiple determinations ($n = 9$ experiments), the mean level of PNMT mRNA in the presence of PACAP plus α-amanitin was approx 2.6-fold greater than in amanitin-treated cultures alone (Table 2). Determinations conducted at intervals over a course of four separate experiments revealed that PACAP influenced the amount of PNMT mRNA up to 24 h, the longest interval examined. The presence of ~2.4-fold more PNMT at these intervals is consistent with the relative induction seen with 1 n*M* PACAP in the Northern analyses (Fig. 1).

Because variability in relative amounts of PNMT mRNA following inhibition with α-amanitin was observed at early time points in some experiments, these studies were repeated using another inhibitor of transcription, DRB (10 μg/mL). Measurements of TH, DBH, PNMT, and GAPDH mRNA were performed at intervals (0, 8, and 16 h) following the addition of DRB in the presence or absence of PACAP (1 n*M*). Slot blots each containing 2.5 μg chromaffin cell total RNA prepared from triplicate cultures, were probed with random primed cDNAs labeled to comparable specific activities and quantified by phosphorimaging scanning (Fig. 4). After 16-h incubation with DRB, the amounts of TH and DBH mRNA were similar

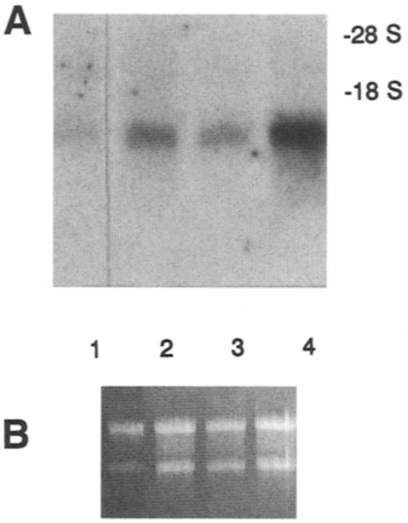

Fig. 3. PACAP stabilizes PNMT mRNA against degradation. Bovine chromaffin cell cultures were treated after 24 h in culture with (1) PBS; (2) PACAP (1 n*M*); (3) α-amanitin (1 μg/mL); and (4) PACAP + α-amanitin. Total RNAs (10 μg) prepared from cultures 16 h later were resolved on denaturing-agarose gels for Northern analysis using PNMT, then 18S rRNA cDNAs as hybridization probes. **(A)** Autoradiogram of PNMT mRNA **(B)** Ethidium bromide staining of total RNA.

or reduced in the presence of PACAP (Fig. 4): TH-9644 vs 9714 and DBH-20,174 vs 17,704 intensity U. The transient increase in DBH mRNA at 8 h was observed in two separate experiments. Although the reason for this increase was not investigated further, it may be indicative that a separate mode of transcription-independent stabilization may additionally influence DBH mRNA levels, possibly through specific cis-active elements encoded in its sequence. However, at both 8 and 16 h, the amounts of PNMT mRNA were 1.5-fold greater in PACAP + DRB than in DRB-treated cultures: 8 h, 8808 vs 5784 and 16 h, 7027 vs 4701 U. Because the levels of PNMT were greater in the presence of PACAP during inhibition of transcription, these results are consistent with a post-transcriptional influence of PACAP, possibly a stabilization of PNMT mRNA against degradation.

Discussion

The adrenal enzymes TH, DBH, and PNMT catalyze the synthesis of norepinephrine and epinephrine, two hormones essential for regulation of blood pressure. Sympathetic tone is mediated largely via neural afferents to the adrenal medulla, and effectively governs both the release and compensatory biosynthesis of catecholamines in this tissue. The presence of the neuropeptide PACAP during splanchnic-mediated neural stimulation, and the ability of this peptide to stimulate release of adrenal catecholamines from chromaffin cells and PC12s, has led to the hypothesis that PACAP may function as a essential component, possibly a "missing, i.e., noncholinergic, link" in the adrenal medullary response to stressful stimuli. It has been postulated that the effects of PACAP could account for elevations of intracellular cAMP observed following stressful stimuli (Przywara et al., 1996), which cannot be attributed to known nicotinic- or muscarinic-receptor mediated events (Strong et al., 1992; Wakade et al., 1992; Watanabe et al., 1992; Rius et al., 1994).

The present study addresses the issue of whether PACAP influences the production of circulating catecholamines by regulating expression of the genes for TH, DBH, and PNMT. In addition to confirming previous reports showing PACAP stimulatory effects on TH mRNA (Rius et al., 1994; May and Braase, 1995), the authors additionally dem-

Table 2
Effects of PACAP on Degradation of PNMT mRNA

Treatment	Amount PNMT mRNA relative to control cultures
Control	1
α-Amanitin	1.15 ± .56[a]
PACAP + α-Amanitin	2.64 ± .93[b]

Bovine chromaffin cell cultures were treated with media alone, α-amanitin (1 μg/mL) or PACAP (1 n*M*) + α-amanitin, for 16 h. Total RNAs were prepared from triplicate cultures for hybridization with PNMT cDNA and 18S rRNA cDNA (for normalization). RNAs were quantified by dot, slot, and Northern blot analyses in a total of nine experiments. Data represent the normalized PNMT autoradiographic densities in treated cultures relative to saline-treated control cultures and are expressed as the mean ±SE. Data pertaining to PNMT mRNA stability in the presence of PACAP were examined for normal and non-Gaussian distribution by the Shapiro-Wilk test. For comparison of two nonnormally distributed groups, the Mann-Whitney rank sum test was used. [a]$p < 0.0005$, [b]$p < 0.0005$.

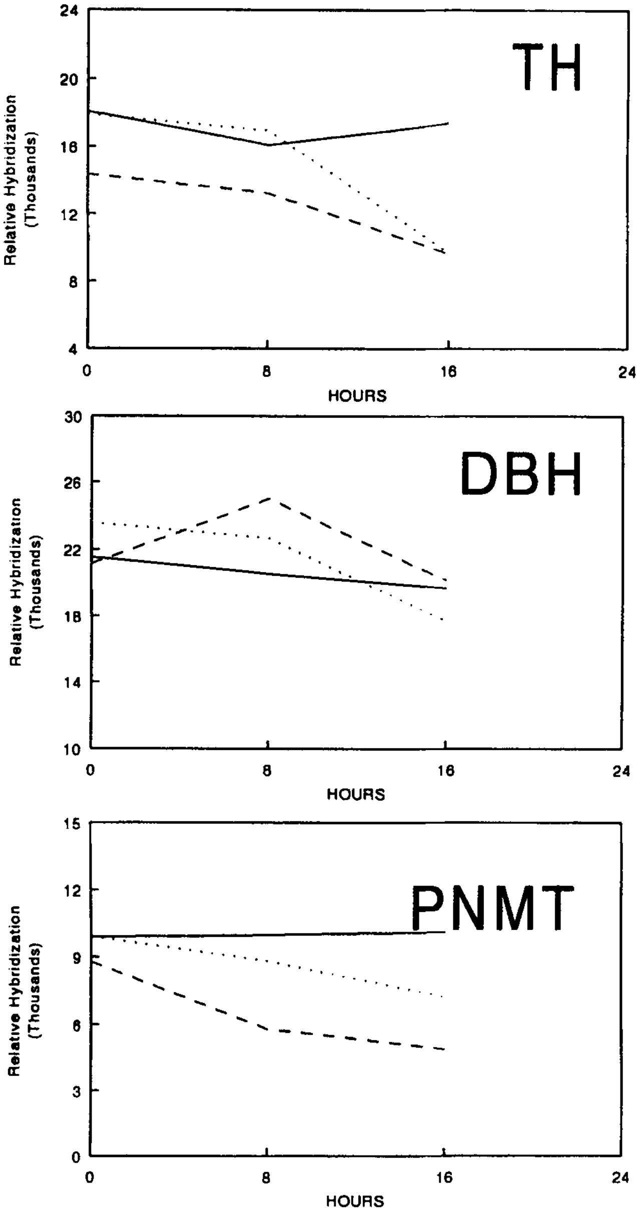

Fig. 4. PACAP Effects on catecholamine enzyme mRNAs following transcriptional inhibition by DRB. Bovine chromaffin cell cultures were treated with DMEM (control; solid line), DRB (10 µg/mL; dashed line), or DRB + PACAP (1 n*M*; dotted line) for 0, 8, or 16 h prior to preparation of total RNA. RNAs were fixed to Gene Screen Plus in quadruplicate (2.5 µg each), then hybridized to ^{32}P-labeled cDNA probes (~5 × 10^8 cpm/µg) for TH, DBH, PNMT, and GAPDH (for normalization). The signals were quantified by scanning with a phosphorimager (ImageQuaNT) and signals expressed as units hybridized.

onstrate that PACAP elevates levels of the mRNAs encoding DBH and PNMT. Evidence supporting a physiological role for this neuropeptide, with regard to production of adrenal catecholamines include: Nanomolar concentrations of PACAP produce ~10-fold elevations of TH and DBH mRNAs and ~2.4-fold rises for PNMT mRNA. This nanomolar potency of PACAP contrasts with the micromolar concentrations of VIP (a peptide sharing 68% sequence similarity) required to elicit catecholamine release (Guo and Wakade, 1994; Chowdhury et al., 1994), or equivalent elevations of cAMP (Deutsch and Sun, 1992), and TH activity (Rius et al., 1994).

Also consistent with a physiological role for PACAP is the observation that the magnitude of its effects on catecholamine enzyme mRNAs is comparable to that elicited by cholinergic agonists and depolarizing effectors. The fact that PACAP is maximally effective at physiological concentrations for all three of the catecholamine synthetic enzyme mRNAs argues that this neuropeptide represents an integral component in the neural regulation of catecholamine biosynthesis in the adrenal medulla.

Although TH enzymatic activity is considered rate-limiting in catecholamine biosynthesis, regulation of the genes encoding each of the catecholamine biosynthetic enzymes is in fact distinctive for selected effectors. Specifically, differing responses are observed for TH and DBH mRNAs with regard to the abilities of nicotine and depolarizing concentrations of K^+ to enhance respective mRNA production (Kilbourne et al., 1992; Sabban and Nankova, 1996). Temporal responses of TH and PNMT mRNAs to clonidine are likewise distinctive (Evinger et al., 1995). Moreover, although the TH and DBH genes contain cAMP-responsive elements (CREs) through which stimulated production of cAMP is postulated to influence gene expression (Sassone-Corsi, 1995), the 5' region of the rat PNMT promoter (Ross et al., 1990) used in the present study does not possess canonical CREs through which cAMP induction characteristically is mediated.

Relative effects of PACAP are specific for each gene and stimulus. In equivalent treatment paradigms, the magnitude of PACAP-mediated effects were greater on DBH and TH mRNAs than were observed for PNMT mRNA, thereby indicating different response characteristics for these genes. The fact that adrenal medullary PACAP is localized only in noradrenergic cells (Shiotani et al., 1995), and that it releases catecholamines in a 7:1 ratio of adrenaline to noradrenaline (Guo and Wakade, 1994), also argues for distinct regulation of the catecholamine biosynthetic enzyme genes by this neuropeptide.

Moreover, the mechanism of PACAP action on expression of the PNMT gene appears to be separate from that observed for glucocorticoid (Ross et al., 1990; Evinger et al., 1992) cholinergic, or depolarizing agents (Evinger et al., 1994), all of which have been shown to stimulate the rate of PNMT gene transcription. In contrast, the present data in this study indicate that PACAP does not exert its effects on PNMT expression by enhancing transcription in the manner seen with other effectors. Specifically, rates of PNMT gene transcription are not altered by 1–10 n*M* PACAP, in contrast to the 2.2–7-fold increases elicited by 50 m*M* K^+ or 3.3-fold changes evoked by muscarine (Table 1). Furthermore, expression of PNMT promoter constructs transfected into bovine chromaffin cells does not change following PACAP treatment using concentrations that evoke two- to threefold increases in endogenous PNMT mRNA levels. That PACAP effects are additive with those of other neural stimuli, e.g., nicotine (Rius et al., 1994), muscarine, and depolarization by 50 m*M* potassium (not shown), is further consistent with suggestions (Wakade et al., 1992; Przywara et al., 1996) that PACAP acts through intracellular mechanisms separate from those of conventional cholinergic stimuli.

Because the authors' transient transfection and in vitro run-on studies exclude a transcriptional effect, the possibility that PACAP exerts its influence on PNMT mRNA steady-state levels posttranscriptionally, was explored by comparing the relative amounts of PNMT message remaining following inhibition of transcription in the presence and absence of PACAP. As α-amanitin had previously been shown to inhibit chromaffin cell transcription by RNA polymerase II (Evinger et al., 1989; Stachowiak et al., 1990), this compound was utilized in initial studies to determine whether PACAP alters the half-life of PNMT mRNA. The amount of PNMT mRNA was two- to fourfold (mean 2.63 ± 0.93) greater in cultures treated with PACAP plus α-amanitin at 16 h. Analogous studies using the transcriptional inhibitor DRB likewise revealed higher levels of PNMT mRNA in PACAP-treated cultures; PACAP had little or no effect on TH or DBH mRNA after 16 h in these DRB-treated cultures. Thus, it is likely that PACAP exerts its effects on PNMT mRNA primarily by altering the rate at which the mRNA is degraded. These data, however, do not distinguish the mechanism (e.g., interaction with specific binding factors, inhibition of specific nucleases, or translational stabilization—reviewed in Hentze, 1991) by which this stabilization may occur.

It is documented that activation of adenylate cyclase by VIP, forskolin, and 8-Br cAMP stimulates TH and DBH expression (Kumakura et al., 1979; Tank et al., 1986; Lewis et al., 1987; Zigmond, 1988; Wessels-Reiker et al., 1993; Kim et al., 1994). Sabban, Tank, and colleagues have shown that nicotine elevates transcription of TH mRNA in PC12 cells through a CRE sequence at –37 to –45 in the 5' regulatory region of this gene (Fossom et al., 1991; Kilbourne et al., 1992). Likewise, DBH gene expression responds to stimulation of cAMP production (McMahon and Sabban, 1992; Kim et al., 1994). These responses are generally attributed to interactions of nuclear transacting proteins, e.g., CREB complexes, with specific sequences, i.e., CREs, encoded in the TH and DBH genes. Thus, because PACAP activates chromaffin cell adenylate cyclase, it is reasonable to hypothesize that PACAP may exert transcriptional effects on the TH and DBH genes through their respective CRE sequences.

In contrast, no canonical CRE sequence has been detected for any of the PNMT genes examined thus far. Thus, it is possible that PACAP influence on PNMT mRNA expression is not mediated through the same protein kinase A pathway by which the TH gene may respond to PACAP. Recent studies have demonstrated that PACAP can activate pathways involving of phospholipase C (Pisegna and Wank, 1996), mitogen-activated protein (MAP) kinase (Villalba et al., 1997), selected calcium dependent kinases, and mitogen-stimulated proliferation of adrenal medullary cells (Tischler et al., 1995). It is therefore, conceivable that the influence of PACAP on PNMT mRNA production occurs though a mechanism entirely distinct from its effects on TH and DBH mRNAs. Comparison of the relative influence of PACAP on expression of the TH, DBH, and PNMT genes may reveal further diversity in neurally mediated regulation of catecholamine biosynthesis in the adrenal medulla.

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