

Pituitary Adenylate Cyclase Activating Polypeptide (PACAP) Expression in Sympathetic Preganglionic Projection Neurons to the Superior Cervical Ganglion

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ABSTRACT: Pituitary adenylate cyclase activating polypeptides (PACAP27 and PACAP38) are members of the VIP/secretin/glucagon family of peptides and have diverse neuroregulatory effects in sympathoadrenal cell development and function. PACAP peptides regulate rat superior cervical ganglion (SCG) neuron catecholamine and neuropeptide Y content and secretion, and promote sympathoneuroblast survival through activation of specific PACAP₁ receptor isoforms. In examining the potential sources of PACAP regulating the SCG, PACAP expression was identified in rat preganglionic neurons in the intermediolateral cell column (IML) of the thoracic spinal cord which provide primary afferent projections to this sympathetic ganglion. Thoracic spinal cord segments (T1–4) contained approximately 17 pmol PACAP38 immunoreactivity/g tissue wet weight. Reverse-transcription polymerase chain reaction of cDNA from microdissected thoracic spinal cord using primers specific for rat neuronal proPACAP identified proPACAP mRNA expression in the IML; the results correlated with neurons labeled for proPACAP mRNA by *in situ* hybridization histochemistry and

implicated PACAP biosynthesis in IML neurons. To demonstrate directly proPACAP transcript expression in preganglionic projection neurons to the SCG, the ganglion was decentralized and the sympathetic trunk immersed in fluorogold to identify sympathetic preganglionic neurons by retrograde labeling. Cryosections of spinal cord segments containing preganglionic neuron fluorogold labeled neurons were processed subsequently for *in situ* hybridization histochemical localization of proPACAP mRNA using a digoxigenin-labeled riboprobe; IML neurons were examined for fluorogold and digoxigenin/alkaline phosphatase product dual labeling. More than half of the preganglionic projection neurons to the SCG expressed PACAP mRNA, consistent with the postulate that PACAP peptides released from a subpopulation of thoracic IML preganglionic neurons may be physiological anterograde modulators of sympathetic SCG function. © 1998 John Wiley & Sons, Inc. *J Neurobiol* 36: 325–336, 1998

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The pituitary adenylate cyclase activating polypeptide (PACAP)/vasoactive intestinal peptide (VIP) family of related peptides has well-established roles

in central and peripheral nervous system function and development. Recently, the neurophysiological effects of PACAP peptides have been shown to overlap significantly with those mediated by VIP. PACAP and VIP receptor sites, for example, have been described throughout the neuraxis (Loren et al., 1977; Martin et al., 1987; Masuo et al., 1992, 1993; Hill et al., 1994; Harmar and Lutz, 1994). In central and peripheral neurons, PACAP and VIP facilitate neuronal depolarization and excitability,

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induce second-messenger production, and increase neurotransmitter levels by augmenting biosynthetic enzyme phosphorylation, activity, and mRNA expression (Phillis, 1982; Ip et al., 1982, 1985; Luine et al., 1984; Schwarzschild et al., 1989; Zigmond et al., 1989; Tatsuno et al., 1992; Braas and May, 1995; Beaudet et al., 1997). The two peptides have been identified in specific sensory and autonomic neurons (Sundler et al., 1988; 1996; Mulder et al., 1994, 1995; Zhang et al., 1995, 1996; Brandenburg et al., 1997) and have been implicated in neuron-mediated cardiovascular vasodilation, gastrointestinal smooth-muscle relaxation, and neuroendocrine hormone secretion (Fahrenkrug, 1993; Arimura and Shioda 1995).

The 175-amino-acid rat PACAP precursor molecule is posttranslationally processed in a tissue-specific manner to produce either of the two biologically active α -amidated products: PACAP38 is a 38-amino-acid form of PACAP [proPACAP(31–168)] and PACAP27 is an alternatively processed form corresponding to the 27 amino terminal residues of PACAP38 [proPACAP(131–157)] (Miyata et al., 1989, 1990; Ogi et al., 1990; Arimura et al., 1991, 1992). PACAP27 shares more than 68% amino acid sequence homology with VIP; there is only one amino acid substitution between amphibian and mammalian PACAP peptides implicating highly conserved functional roles throughout evolution (Chartrel et al., 1991; Ohkubo et al., 1992; Okazaki et al., 1995; Rawlings and Hezareh, 1996). The effects of VIP and PACAP on target cells are mediated by at least three putative seven-transmembrane G protein-coupled receptor subtypes identified to date (Christophe, 1993; Spengler et al., 1993; Harmar and Lutz, 1994; Journot et al., 1994, 1995; Rawlings and Hezareh, 1966). The two PACAP peptides are several orders of magnitude more potent than VIP in stimulating second-messenger activation at the PACAP-selective (PACAP₁) receptor; in contrast, the VIP₁/PACAP and VIP₂/PACAP receptors exhibit approximate equal high affinity for PACAP27, PACAP38, and VIP, and may represent the prototypic VIP receptors (Christophe, 1993; Harmar and Lutz, 1994; Journot et al., 1995; Arimura and Shioda, 1995).

Among the many neurophysiological roles, PACAP peptides exhibit diverse functions in autonomic development and function. In the sympathetic nervous system, PACAP peptides are potent and efficacious regulators of sympathetic superior cervical ganglion (SCG) neurotransmitter and neuropeptide expression. In accord with the predicted pharmacological response profile for the PACAP₁ receptor, PACAP27 and PACAP38 were at least

100-fold more potent than VIP in stimulating neuropeptide Y (NPY) and catecholamine release in primary SCG neuronal cultures (May and Braas, 1995; Braas and May, 1996). The stimulatory effects were sustained in concert with increased neuropeptide and neurotransmitter production, and elevated pro-NPY and tyrosine hydroxylase mRNA expression. Reverse-transcription polymerase chain reaction (PCR) using SCG cDNA templates and primers specific to the PACAP-selective receptor demonstrated high levels of PACAP₁ receptor expression; *in situ* hybridization studies using riboprobes to the receptor suggested that the majority of principal sympathetic neurons expressed the PACAP₁ receptor (May and Braas, 1995; Beaudet et al., 1997; May et al., 1997). PACAP was also more potent than VIP in stimulating primary adrenal medullary chromaffin and PC12 pheochromocytoma cells (Deutsch and Sun, 1992; Isobe et al., 1993; Watanabe et al., 1995) and in enhancing sympathoneuroblast survival and mitosis (DiCicco-Bloom and Deutsch, 1992). The potency of PACAP peptides in stimulating sympathetic neurons, the prevalence of the PACAP-selective receptor expression in the SCG, and the prominent roles for PACAP in neuroblast maturation may position PACAP peptides uniquely among the physiological noncholinergic regulators of sympathoadrenal cell development and function.

The sympathetic neurons of the rat SCG are regulated primarily by preganglionic neurons located in the intermediolateral cell column (IML) of the thoracic spinal cord. Although acetylcholine has been regarded classically as the primary anterograde regulator of sympathetic neurons, there is substantial evidence for noncholinergic preganglionic neurotransmitter action in the rat SCG (Ip et al., 1982, 1983, 1985; Schwarzschild et al., 1989; Zigmond et al., 1989). Given the functional scope of PACAP peptides in the sympathetic ganglia, the present studies were performed to identify the potential sites of PACAP production that regulate the SCG with the principal hypothesis that sympathetic preganglionic projection neurons from the spinal cord IML represented a physiologically relevant source of PACAP peptides that guide sympathetic postganglionic neuronal function. Identification of PACAP₁ receptor expression in the SCG established mechanisms for PACAP-selective effects. PACAP immunoreactivity and mRNA expression were examined in thoracic spinal cord segments and PACAP-expressing neurons were identified in the IML; combined retrograde labeling and *in situ* hybridization studies demonstrated that PACAP-containing upper thoracic IML preganglionic neurons have direct fiber projec-

tions to SCG neurons. These results are consistent with the postulate that PACAP peptides in IML preganglionic neurons may represent key antero-grade regulatory signals of postganglionic sympathetic SCG function.

MATERIALS AND METHODS

Animals

Superior cervical ganglion and thoracic spinal cord tissues (T1–4 segments) from decapitated adult male Sprague–Dawley rats (225–250 g; Charles River, Canada) were stored at -80°C until extraction for peptide immunoassays or total RNA. Additional tissue samples from rats of the same weight and strain were obtained from Zivic-Miller Laboratories (Zelienople, PA); the tissues were snap-frozen in liquid nitrogen, shipped on dry ice, and stored at -80°C until tissue preparation.

Radioimmunoassay

Radioimmunoassays were performed as previously described (Braas et al., 1994a, 1994b; May et al., 1995; May and Braas, 1995; Brandenburg et al., 1997). Spinal cord tissues were extracted in 5 *N* acetic acid containing 2 mg/mL bovine serum albumin (BSA) and 0.3 mg/mL phenylmethylsulfonyl fluoride (PMSF), lyophilized, and resuspended in 100 mM sodium phosphate buffer, pH 7.5, containing 1% Triton X-100 and 0.3 mg/mL PMSF. Tissue PACAP38 levels were determined by double-antibody radioimmunoassays (Peninsula Laboratories, Belmont, CA) (Brandenburg et al., 1997).

Thoracic Spinal Cord Micropunches

Tissue blocks containing rat thoracic spinal cord segments T1–4 were placed in Tissue Tek OCT compound (Miles Inc., Elkhart, IN) and frozen in a dry ice/alcohol slurry. Cryosections (400 μm) were prepared on a freezing microtome and mounted onto subbed slides. The slides were placed on a bed of crushed dry ice for examination under a dissecting microscope, and the frozen tissue sections were microdissected using a 0.3-mm-diameter punch (Stoelting, Wood Dale, IL). Bilateral punches were prepared from the dorsal horn, ventral horn, IML, or white matter according to Palkovits and Brownstein (1988); corresponding punches from four consecutive tissue sections (eight punches total) were pooled for RNA extraction and reverse-transcription PCR. Two to three independent pools of tissues were prepared from each thoracic spinal cord region. DNA templates from each spinal cord region were examined for proPACAP, choline acetyltransferase, and β -actin mRNA expression.

Reverse-Transcription PCR and Sequence-Specific Hybridization

Total RNA from spinal cord segments or micropunches, hypothalamus, anterior pituitary gland, or SCG was prepared using the RNA STAT-60 total RNA/mRNA isolation reagent (Tel-Test B, Friendswood, TX) as previously described (Braas et al., 1994a, 1994b; May and Braas, 1995; Brandenburg et al., 1997). First-strand cDNA was produced using SuperScript II reverse transcriptase and oligo-dT primers using the SuperScript Preamplification System (Gibco-BRL Life Technologies, Grand Island, NY). Single-stranded cDNA was amplified by PCR using primers specific for rat neuronal PACAP, PACAP₁ receptor, choline acetyltransferase, or β -actin (Table 1) (May and Braas, 1995; Brandenburg et al., 1997). Amplified products were resolved on 1.6% agarose gels and visualized by ethidium bromide staining under ultraviolet (UV) illumination.

Verification of the reverse-transcription proPACAP PCR products was performed using sequence-specific hybridization (Brandenburg et al., 1997). The amplified DNA, fractionated on 1.6% agarose gels, was denatured, neutralized, and rapidly downward-transferred to Nytran-Plus membrane (Schleicher and Schuell, Keene, NH). The membranes were prehybridized in $1.5\times$ SSPE (225 mM NaCl, 15 mM monobasic sodium phosphate, and 1.5 mM EDTA; GIBCO-BRL Life Technologies) containing 10% polyethylene glycol, 7% sodium dodecyl sulfate, and 200 $\mu\text{g}/\text{ml}$ salmon sperm DNA, and hybridized at 65°C for 48 h with the synthetic antisense internal oligonucleotide probe, 5'-TGGTCTGATCCCAGGGAAGCTGAGTCCGGCGGCAGGTGAACA-3', end-labeled with [^{32}P] γ ATP using T4 polynucleotide kinase (Promega, Madison, WI). The blots were washed and apposed to Reflection autoradiographic film (NEN, Boston, MA).

In Situ Hybridization Histochemistry

Cryosections of spinal cord T1–4 segments were fixed in 4% paraformaldehyde, washed, acetylated, and hybridized with either digoxigenin- or [^{35}S]CTP-labeled PACAP riboprobes for 24 h at 45°C (Braas et al., 1994b; Brandenburg et al., 1997). A 606-base-pair fragment of rat proPACAP cDNA (nucleotides 486–1071; Genbank M63006) was subcloned into pBlue-script II KS⁻ (Stratagene, La Jolla, CA) to generate antisense and sense riboprobes (Brandenburg et al., 1997). Sections hybridized with digoxigenin probes (Genius System; Boehringer Mannheim, Indianapolis, IN) were processed with anti-digoxigenin-alkaline phosphatase using Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate as phosphatase substrates in the presence of the isoform-selective alkaline phosphatase inhibitor levamisole. Sections hybridized with radiolabeled riboprobes were exposed to Kodak NTB-2 emulsion for 5–7 days and processed for visualization under dark-field illumination.

Table 1 Reverse-Transcription Polymerase Chain Reaction Gene-Specific Primers

Specificity	Sequence	Position*	Annealing Temperature	Product Size (bp)
Neuronal PACAP transcript	5'-ATGCCTCTCTGGTTGTGATTC-3'	486–506	57°C	606
	5'-CGCTATTCGGCGTCTTTGTT-3'	1071–1091		
PACAP ₁ receptor	5'-CACAGTATTCGCCTTCTCTCC-3'	1185–1205	56°C	449
	5'-GCCTATCCCTATCTCTCTTT-3'	1613–1633		
Choline acetyltransferase	5'-CCTCATCTCTGGTGTGCTTAG-3'	588–608	56°C	690
	5'-TTCTTGTTGCTTGTCATCATA-3'	1257–1277		
VIP	5'-AACAAACCAAGTGATTACATTA-3'	2725–2750	49°C	672
	5'-GTGAGTGATTACGGAAAAGAGT-3'			
β -actin	5'-TCATGAAGTGTGACGTTGACATCCGT-3'	2725–2750	59°C	285
	5'-CCTAGAAGCATTGCGGTGCACGATG-3'	3108–3133		

* GenBank accession numbers: neuronal PACAP transcript, M63006; PACAP₁ receptor, Z23279; β -actin, J00691. Choline acetyltransferase sequence from Brice et al., *J. Neurosci. Res.* **23**:266–273; VIP sequence from Giladi et al., *Brain Res. Mol. Brain Res.* **7**:261–267.

Surgical Procedure and Retrograde Labeling

Retrograde labeling of preganglionic neurons to the SCG was performed by Zivic Miller Laboratories according to procedures described by Baldwin et al. (1991). Cervical sympathetic trunks (CST) of anesthetized adult male Sprague–Dawley rats were exposed bilaterally; one CST was transected 1–2 mm below the SCG and the exposed contralateral trunk served as sham control. The distal end of the transected CST was placed in a paraffin well containing 2–3 μ L of 4% fluorogold (Fluorochrome, Englewood, CO); the animal was placed under a warming lamp and a saline-dampened sterile gauze was placed over the surgical site. After retrograde labeling for 30 min, the incisions were closed with surgical staples and the animals were allowed to recover for 7 days. Subsequently, the animals were decapitated and the dissected thoracic spinal cord segments T1–4 were snap-frozen in liquid nitrogen for delivery on dry ice.

To identify the labeled preganglionic neurons, the frozen tissues were embedded subsequently in Tissue Tek OCT compound and placed in a dry ice/alcohol slurry. Cryosections (25 μ m) of the thoracic cord were mounted on sterile subbed slides, fixed in 4% paraformaldehyde containing 0.1% glutaraldehyde, and examined by fluorescence microscopy under UV illumination. Sections with fluorogold-labeled IML preganglionic neurons projecting to the SCG were photographed; the coverslips were removed and the slides were rinsed in 2 \times SSC and processed for *in situ* hybridization histochemical localization of proPACAP mRNA. Fluorogold-labeled neurons were observed unilaterally and confined to the IML; the contralateral IML was always devoid of retrograde staining. Postfixation of the cryostat tissue sections, which produced superior *in situ* hybridization histochemical localization of proPACAP transcripts, produced slight diffusion of the fluorogold around the labeled neurons, but did not compromise neuronal identification under fluorescence microscopy.

RESULTS

PACAP Peptides and mRNA Are Expressed in Thoracic Spinal Cord

By any measure, including stimulation of sympathetic neuronal transmitter and peptide secretion, second-messenger activation and transmitter biosynthetic enzyme mRNA expression, the pharmacological response profiles of postganglionic sympathetic neuron to PACAP and VIP peptides indicated that the SCG express predominantly the PACAP₁ receptor (Braas and May, 1994, 1996; May and Braas, 1995; Beaudet et al., 1997). An essential requirement for the observed PACAP-mediated effects must include the demonstration of PACAP-selective receptors in the sympathetic ganglia. Reverse-transcription PCR of adult rat SCG templates using oligonucleotide primers to amplify a segment of DNA corresponding to the intracellular cytoplasmic tail of the receptor detected readily mRNA encoding the PACAP₁ receptor in the SCG [Fig. 1(A)]. A single amplified 449-base-pair product identical in size to that predicted from the known rat PACAP₁ receptor sequence was observed; the same-sized product was identified also in preparations from hypothalamus and anterior pituitary gland, two tissues with high levels of PACAP₁ receptor expression (Spengler et al., 1993). More detailed studies with SCG preparations indicated that sympathetic neurons did not express substantial levels of either the nonselective VIP₁/PACAP or VIP₂/PACAP receptor mRNA, suggesting that the sympathetic neuronal responses are mediated primarily by the PACAP-selective PACAP₁ receptor subtype rather than the nonselective VIP/PACAP receptors (Braas and May, 1994, 1996, data not shown).

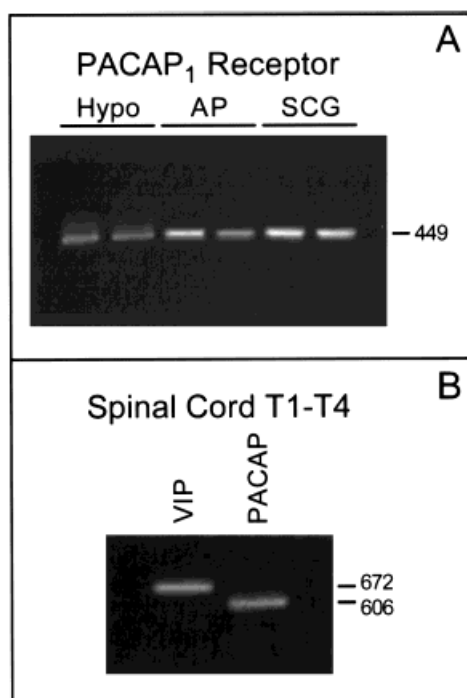


Figure 1 Thoracic spinal cord and SCG express PACAP and PACAP₁ receptor mRNA, respectively. (A) Reverse-transcribed cDNA templates from total SCG, hypothalamus (Hypo) anterior pituitary gland (AP), and RNA were amplified using primers to a 449-base-pair region within the carboxyl terminus of the PACAP₁ receptor transcript (see Table 1). Each lane represents an independent animal tissue preparation. (B) Total RNA from thoracic spinal cord tissue was reverse-transcribed and amplified using primers specific for rat proPACAP or proVIP. The predicted product sizes are 606 base pairs for proPACAP and 672 base pairs for proVIP. The amplified products were resolved on 1.6% agarose gels, stained with ethidium bromide, and visualized by UV illumination.

These results provided the impetus to examine the endogenous sources of PACAP that interact with SCG PACAP₁ receptors.

The anterograde signals that regulate sympathetic neurons arise primarily from preganglionic neurons in the IML of thoracic and lumbar spinal cord segments. As a necessary requisite, that supposition implied that PACAP peptide immunoreactivity in either neuronal soma or fibers must be present at quantitatively appreciable levels in these particular spinal cord segments. Accordingly, PACAP peptides in thoracic spinal cord T1–4 segments containing preganglionic projection neurons to the SCG were measured as an initial approximation. The thoracic spinal cord segments contained significant levels of PACAP immunoreactivity. When spinal cord segments T1–4 were extracted for PACAP38 radio-

immunoassay, the samples contained a total of 1.2 ± 0.1 pmol of PACAP38-immunoreactive material which represented 17 ± 0.8 pmol of immunoreactive peptide/g tissue wet weight [77 ng peptide/g tissue wet weight; mean \pm standard error or the mean (SEM), $n = 5$].

Although these results were requirements for endogenous spinal cord PACAP production, the data did not preclude the possibility that the measurements solely represented peptide immunoreactivity in fibers or terminals of extrinsic derivation. For intrinsic PACAP production, PACAP mRNA transcripts must be present in the same spinal cord segments examined for PACAP peptide immunoreactivity. Consequently, a reverse-transcription PCR-based approach was undertaken to assess the endogenous expression of PACAP mRNA necessary for PACAP biosynthesis in the thoracic cord segments. Reverse transcription PCR using T1–4 spinal cord DNA templates produced the anticipated 606-base-pair product for proPACAP [Fig. 1(B)]. The same amplified product was identified in hypothalamus and other neuronal tissues of high PACAP expression (Brandenburg et al., 1997). Previous studies suggested VIP expression in thoracic cord may participate in SCG regulation (Sasek et al., 1991; Baldwin et al., 1991); amplification of the same thoracic spinal cord cDNA with primers for rat proVIP produced a 672-base-pair product. Although these results did not discern either the distribution or relative contributions of PACAP and VIP in SCG regulation, these whole-tissue studies demonstrated the potential for endogenous PACAP synthesis in thoracic spinal segments and provided a basis for more detailed investigations of PACAP expression in the IML.

PACAP Peptides Are Expressed Predominantly in the Intermediolateral Cell Column of the Spinal Cord

To more accurately ascribe PACAP mRNA expression to specific regions of the spinal cord regions, micropunched tissue samples from the IML, dorsal horn, ventral horn, and white matter of T1–4 spinal cord were extracted for total RNA and reverse-transcription PCR (Fig. 2). Amplification of microdissected samples using oligonucleotide primers specific for proPACAP demonstrated proPACAP transcript expression in the intermediolateral grey column. The presence of proPACAP mRNA was also observed in dorsal horn cells; PACAP mRNA was lower in ventral horn and not detectable in white matter. The identity of the PACAP PCR products in the micropunched spinal cord samples was

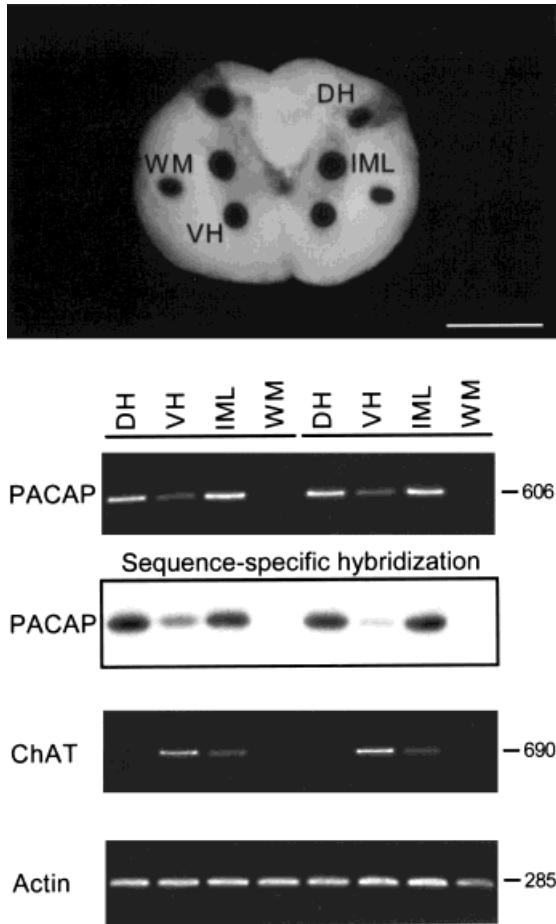


Figure 2 ProPACAP mRNA is expressed in sympathetic preganglionic neurons. Spinal cord frozen sections (400 μm) were microdissected using a 0.3-mm brain punch. Spinal cord microdissected regions are shown in the top panel. Total RNA from intermediolateral cell column (IML), dorsal horn (DH), ventral horn (VH), and white matter (WM) was prepared for reverse-transcription PCR using primers for rat neuronal PACAP, choline acetyltransferase (ChAT), or β -actin. The amplified products for PACAP were transferred to Nytran Plus membrane for sequence-specific hybridization using a [^{32}P]-radiolabeled internal oligonucleotide probe. The two sets of spinal cord tissues (DH, VH, IML, and WM) represent two independent preparations of micropunched tissues. The predicted product sizes are 606 base pairs for PACAP, 690 base pairs for ChAT, and 285 base pairs for β -actin.

verified by sequence-specific hybridization (Fig. 2). The products amplified with the proPACAP primers were transferred to Nytran, denatured, and hybridized to a radiolabeled synthetic antisense oligonucleotide probe that recognized a sequence internal to the primer sites. Hybridization of the probe to the amplified products from intermediolateral cell column, dorsal horn, and ventral horn produced a

single band of the expected size, further establishing the expression of proPACAP mRNA in the cord preparations (Fig. 2); no hybridization signal was observed in white matter even under prolonged film exposure.

To further substantiate that PACAP mRNA expression was region selective, the same cDNA templates from the micropunched tissue samples were amplified for choline acetyltransferase mRNA expression. The tissue patterns for choline acetyltransferase mRNA expression was different from those for PACAP transcripts; the levels of the 690-base-pair-amplified product for choline acetyltransferase mRNA was highest in ventral horn with lower levels in the IML as expected for the expression of the biosynthetic enzyme in the two regions. As anticipated, choline acetyltransferase mRNA expression was not detected in either dorsal horn or white matter. Amplification using primers for β -actin mRNA demonstrated uniform levels of expression in all of the micropunched tissue samples.

To definitively identify the spinal cord nuclei expressing PACAP mRNA, spinal cord cryosections were prepared for *in situ* hybridization (Fig. 3). The *in situ* hybridization histochemical results for PACAP mRNA in the spinal cord were congruous with data for PACAP peptides by radioimmunoassay and transcripts using reverse-transcription PCR. Using a radiolabeled antisense riboprobe, neurons in the IML demonstrated striking and prominent labeling for proPACAP mRNA (Fig. 3). A subpopulation of neurons in the column demonstrated strong autoradiographic grain densities suggesting that the preganglionic neurons that regulate autonomic function have the potential to express significant levels of PACAP. High labeling densities were observed in the substantia gelatinosa corresponding to laminae I and II of the dorsal horn; a few isolated neurons in the nucleus proprius and lateral spinal nucleus were also labeled (Fig. 3). Although occasional scattered neurons in the intermediate grey, ventral horn, and region adjacent to the central canal also appeared to express PACAP mRNA, grain density levels were much lower.

Thoracic PACAP-Containing IML Preganglionic Neurons Project to the SCG

The identification of PACAP mRNA expression in the intermediolateral cell column of the spinal cord was in concurrence with preganglionic PACAP regulation of sympathetic postganglionic neurons. As a means of testing whether IML preganglionic neurons projecting specifically to the SCG express PA-

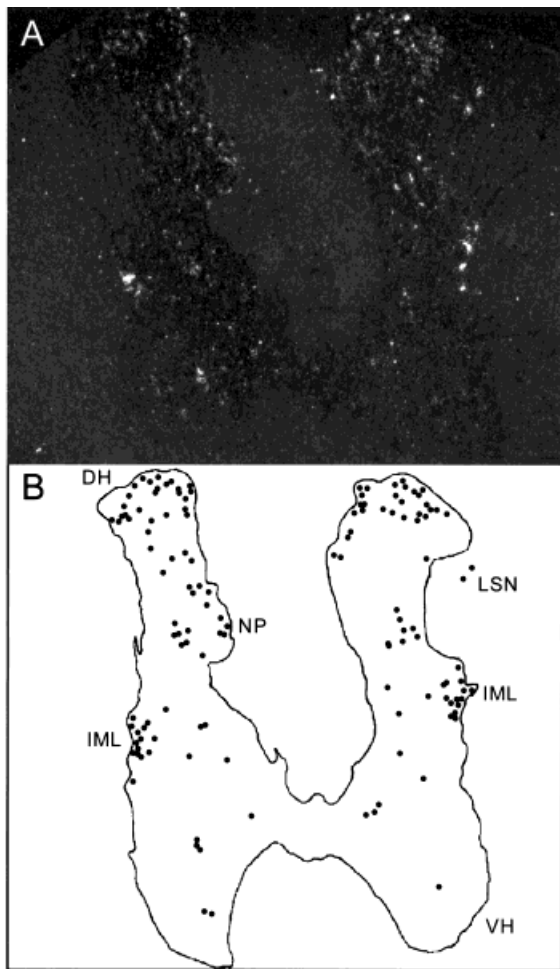


Figure 3 IML neurons express proPACAP mRNA. (A) Spinal cord cryosections from levels T1 to T4 were hybridized with [³⁵S]dCTP-radiolabeled proPACAP riboprobe, exposed to nuclear track emulsion for 5 days, and visualized under dark-field microscopy. (B) Composite camera lucida drawing for PACAP mRNA-labeled neurons from three cryosections. DH = dorsal horn; VH = ventral horn; IML = intermediolateral cell column; WM = white matter; SG = substantia gelatinosa; NP = nucleus proprius; LSN = lateral spinal nucleus.

CAP, a combined retrograde labeling and *in situ* hybridization histochemical protocol was initiated to study proPACAP mRNA expression in the preganglionic neurons that project to the SCG. The CST was transected inferiorly to the SCG and placed in fluorogold for retrograde transport to preganglionic IML neurons that project to the ganglion. These neurons were identified and photographed under UV fluorescence microscopy, and the same tissue sections were processed for PACAP *in situ* hybridization histochemistry using a digoxigenin-labeled proPACAP riboprobe. The same tissue sections regions were rephotographed; the fluoro-

gold- and digoxigenin-labeled cells were compared for coincidence. Fluorogold-labeled preganglionic neurons projecting to the SCG containing PACAP mRNA expression were identified (Fig. 4). Of the retrogradely labeled preganglionic neurons examined ($n = 134$), more than half were labeled also for proPACAP mRNA. *In situ* hybridization labeling using radiolabeled PACAP riboprobes on adjacent thoracic spinal cord sections did not demonstrate differences in either labeling density or the number of labeled IML neurons between the transected CST and sham control halves of the spinal cord. Accordingly, under these experimental paradigms, the observed proPACAP mRNA expression in the IML preganglionic SCG projection neurons could not be attributed solely to an injury response. These results suggested that preganglionic neurons projecting to the SCG can produce endogenously PACAP peptides among many diverse physiological neuroregulatory signals to guide sympathetic function.

DISCUSSION

Although previous work has shown sympathetic SCG neuronal responses to VIP at micromolar concentrations, more recent studies demonstrating the ability for PACAP peptides to stimulate potently sympathetic neuron neurotransmitter/neuropeptide and second-messenger production have suggested that many of the neurophysiological responses attributed previously to VIP may be mediated largely by PACAP. Consistent with that postulate, the majority of sympathetic SCG neurons express preferentially the PACAP-selective PACAP₁ receptor than either the nonselective VIP₁/PACAP or the VIP₂/PACAP receptors. Nevertheless, the potential and physiologically relevant sources of PACAP peptides that regulate sympathetic neurons remained unclear, but included several possibilities such as spinal cord preganglionic neurons, sensory neuronal afferents, endogenous production by postganglionic neurons, and plasma peptides released from neuroendocrine tissues. While plasma in the hypophyseal portal system contained measurable levels of PACAP, free peptide levels in the peripheral circulatory system appeared to be insufficient to mediate neuronal responses (Dow et al., 1994). Similarly, PACAP peptide levels produced endogenously in a small population of SCG sympathetic neurons were several orders of magnitude lower than NPY and would most likely not elicit broad paracrine functions unless induced dramatically under stimulatory physiological paradigms (Kli-

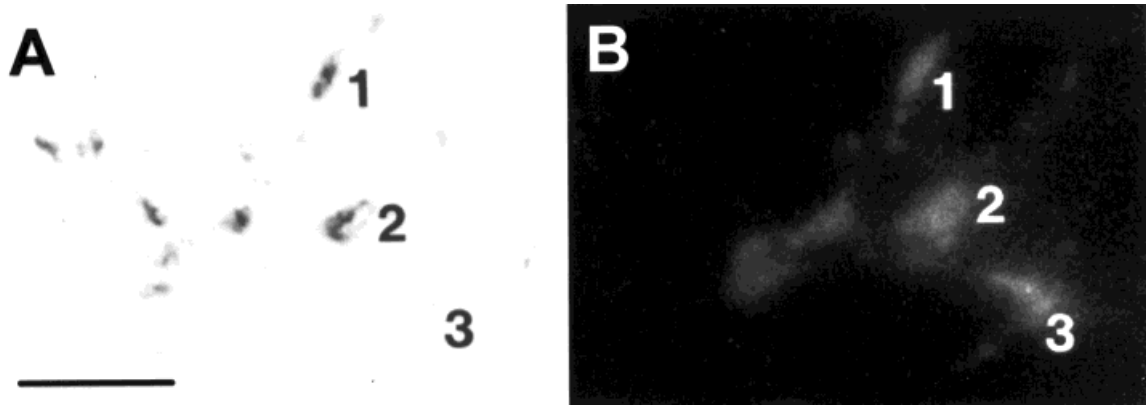


Figure 4 Subpopulations of IML projection neurons to the SCG express proPACAP transcripts. The cervical sympathetic trunk was transected unilaterally 3 mm below the SCG and the preganglionic neurons in the thoracic spinal cord segments projecting to the sympathetic ganglia were retrogradely labeled with fluorogold for sequential fluorescence microscopy and PACAP *in situ* hybridization histochemistry. (B) Cryosections of thoracic spinal cord were photographed under UV illumination to identify fluorogold-labeled neurons in the intermediolateral cell column. (A) The same sections were processed for *in situ* hybridization histochemistry using digoxigenin-labeled PACAP riboprobes. The sections were incubated with alkaline phosphatase-conjugated secondary antisera and processed using Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate as substrates. IML regions were rephotographed under bright-field microscopy; corresponding neurons in the fluorescence and brightfield micrographs were identified and examined for coincidence of fluorogold and digoxigenin labeling. In this example, of the three representative fluorogold retrogradely labeled neurons [neurons 1, 2, and 3 in (B)], only two neurons demonstrated PACAP mRNA expression by *in situ* hybridization histochemistry [neurons 1 and 2 in (A)]. Representative of data from three animals. Scale bar = 100 μm .

maschewski et al., 1996; Brandenburg et al., 1997; Moller et al., 1997a). A small number of dorsal root ganglion neurons contain PACAP peptides and some sensory afferents with PACAP immunoreactivity have been suggested to pervade sympathetic ganglia (Mulder et al., 1994; Sundler et al., 1996). However, the primary anterograde regulation of sympathetic postganglionic neurons is derived from preganglionic neurons in the IML of the thoracolumbar spinal cord, and the presence of PACAP peptides in preganglionic fibers would represent an important mechanism of regulating a major population of sympathetic neurons expressing the PACAP₁ receptor. Postganglionic sympathetic neurons have been well demonstrated to be regulated in part by an anterograde noncholinergic transmitter (Ip et al., 1982, 1983). The increase in postganglionic neuron DOPA synthesis in response to cervical sympathetic trunk stimulation was only reduced approximately 50% in the presence of nicotinic and muscarinic receptor antagonists (Ip et al., 1983) and the remaining noncholinergic stimulatory factor was hypothesized to belong to the VIP family of peptides (Ip et al., 1982, 1983, 1985; Schwarzschild et al., 1989; Zigmond et al., 1989); VIP immunoreactivity

was localized subsequently to a small population of preganglionic neurons implicating potential putative roles as a preganglionic transmitter (Baldwin et al., 1991; Sasek et al., 1991).

The selective expression of the PACAP₁ receptor in the SCG and the current studies demonstrating that a subpopulation of preganglionic neurons projecting to the sympathetic neurons express proPACAP mRNA suggested that PACAP peptides may have a significant, if not a larger, role than VIP in directing sympathetic neuronal responses to anterograde signals. Using several methodological approaches, we demonstrated that spinal cord segments with preganglionic neurons contained PACAP immunoreactivity and mRNA, that preganglionic neurons in the intermediolateral cell columns possessed proPACAP transcripts, and that retrogradely labeled preganglionic neurons projecting to the SCG expressed proPACAP mRNA. The levels of PACAP38 peptide in the thoracic cord were comparable to those found in rat brain cerebral cortex and hippocampus, but an order of magnitude lower than levels present in hypothalamic tissues (Arimura et al., 1991). The identification of PACAP mRNA expression in IML preganglionic neu-

rons by *in situ* hybridization histochemistry was used as an indicator of neuropeptide biosynthetic capability, although the number of neurons in the IML that coexpress PACAP immunoreactivity has not been examined. The present data were in good agreement with more recent supporting physiological data. PACAP-immunoreactive fibers were shown to form dense basket-like plexuses around individual SCG neurons (Sundler et al., 1996; Moller et al., 1997a) and decentralization of the SCG resulted in the abolition of PACAP-immunoreactive fiber staining in the ganglion (Sundler et al., 1996; Moller et al., 1997b). PACAP regulated adrenal medullary and PC12 pheochromocytoma cells (Deutsch and Sun, 1992; Isobe et al., 1993; Ghatei et al., 1993; Watanabe et al., 1995; Holgert et al., 1996; Barrie et al., 1997) and PACAP immunoreactivity was identified in preganglionic splanchnic nerves (Holgert et al., 1996). In this regard, the PACAP peptides may represent a unique peptidergic developmental and functional regulator of cells derived from the sympathoadrenal lineage.

The relative contributions of VIP in regulating sympathetic neurons remain unclear. VIP levels in the IML preganglionic neurons have not been determined; high levels of VIP peptides at localized regions in the SCG may be functionally relevant in activating the PACAP₁ receptor. Alternatively, VIP may interact with a yet unidentified VIP-selective receptor or subserve yet unspecified functions. Nevertheless, the *in situ* hybridization histochemical pattern for PACAP was similar to VIP immunocytochemical staining in the thoracic cord (Sasek et al., 1991). PACAP mRNA and VIP immunoreactivity were identified in the IML; as with previous interpretations, the localization of PACAP and VIP in both fibers and soma in the substantia gelatinosa, and in neuronal soma in the nucleus proprius, suggested that PACAP and VIP may participate in the transmission and processing of primary sensory information (Sasek et al., 1991; Sundler et al., 1996; Dickinson et al., 1997). Although PACAP and VIP appeared to be differentially expressed in central neuronal circuits (Koves et al., 1991; Masuo et al., 1993), the similarities in the distribution pattern of PACAP and VIP in the spinal cord may not be atypical. Both PACAP and VIP were shown to be coexpressed in parasympathetic ganglia and in peripheral fiber systems (Sundler et al., 1996; Hauser-Kronberger et al., 1996); whether PACAP and VIP are colocalized in the same spinal neurons, however, is still unclear.

Recently, PACAP immunoreactivity has also been described in long descending neuronal fibers that appear to innervate the spinal cord intermedio-

lateral preganglionic neurons (Chiba et al., 1996; Dun et al., 1996). The same studies, however did not identify PACAP-related material in the preganglionic neuronal soma which may have reflected the relative abundance of the peptides at different cellular sites. Immunocytochemical localization of peptides represents a balance of parameters including peptide synthesis, storage, and secretion; the relative contribution of each of these parameters in the neuropeptide biosynthetic and secretory process will ultimately describe the specific tissue and cellular staining patterns. If peptide levels are below the limits of detection owing to low levels of expression especially during development, heightened secretion, or absence of a regulated secretory pathway, immunocytochemical methods may be unsatisfactory. In these instances, *in situ* hybridization histochemistry provides a direct means of assessing gene expression and clarifying sites of synthesis when immunological demonstrations fail (Pintar and Lugo, 1987). Since immunoreactive neuropeptide levels are typically higher in nerve fibers and terminals compared to cell bodies, the PACAP data from the previous studies may have been biased to favor localization in fiber tracts.

In summary, the current data demonstrating PACAP expression in IML preganglionic neurons projecting the SCG complement previous studies demonstrating the high potency of PACAP peptides in eliciting sympathetic neuronal effects and PACAP₁ receptors in the SCG, and implicate a neuroregulatory or modulatory role for PACAP in sympathetic function. In this regard, PACAP may be one of many key regulatory signals, including growth factors and cytokines, that guide sympathetic neuronal development, maturation, and responses to autonomic control.

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