Pituitary adenylate cyclase-activating polypeptides directly stimulate sympathetic neuron neuropeptide Y release through PAC1 receptor isoform activation of specific intracellular signaling pathways*

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Pituitary adenylate cyclase-activating polypeptides (PACAP) have potent regulatory and neurotrophic activities on superior cervical ganglion (SCG) sympathetic neurons with pharmacological profiles consistent for the PACAP-selective PAC1 receptor. Multiple PAC1 receptor isoforms are suggested to determine differential peptide potency and receptor coupling to multiple intracellular signaling pathways. The current studies examined rat SCG PAC1 receptor splice variant expression and coupling to intracellular signaling pathways mediating PACAP-stimulated peptide release. PAC1 receptor mRNA was localized in over 90% of SCG neurons, which correlated with the cells expressing receptor protein. The neurons expressed the PAC1 (short) HOP1 receptor but not VIP/PACAP-nonselective VPAC1 receptors; low VPAC2 receptor mRNA levels were restricted to ganglionic nonneuronal cells. PACAP27 and PACAP38 potently and efficaciously stimulated both cAMP and inositol phosphate production; inhibition of phospholipase C augmented PACAP-stimulated cAMP production, but inhibition of adenyl cyclase did not alter stimulated inositol phosphate production. Phospholipase C inhibition blunted neuron peptide release, suggesting that the phosphatidylinositol pathway was a prominent component of the secretory response. These studies demonstrate preferential sympathetic neuron expression of PACAP-selective receptor variants contributing to regulation of autonomic function.

The identification of pituitary adenylate cyclase-activating polypeptide (PACAP)1 and vasoactive intestinal peptide (VIP)/PACAP receptors has broadened our understanding of the mechanisms underlying the regulatory and neurotrophic roles of this family of peptides. The PACAP precursor molecule is tissue-specifically posttranslationally processed to two biologically active a-amidated products, PACAP38 (pro-PACAP-(131–168)) and PACAP27 (pro-PACAP-(131–157)) (1–5), which share amino acid homology with VIP. In the nervous system, PACAP induces neuronal calcium flux, facilitates membrane depolarization, and increases spike frequency (6–8, 10).2 PACAP peptides also enhance neuroblast survival, proliferation, differentiation, and neurite outgrowth and prevent neuronal apoptosis upon growth factor or stimulus withdrawal and ischemic insult (11–16).

The cloning of cDNAs for three putative seven-transmembrane G-protein-coupled receptors for VIP and PACAP demonstrated receptor subtype diversity and functional heterogeneity (17–25). The PACAP-selective PAC1 receptor demonstrates high affinity for only PACAP38 and PACAP27 and is coupled to multiple intracellular signaling cascades. The VPAC1 and VPAC2 receptors, in contrast, exhibit approximate equal high affinity for the PACAP38, PACAP27, and VIP peptides and are coupled to adenylyl cyclase. Multiple PAC1 receptor isoforms result from the alternative splicing of two exons in the aminoterminal extracellular domain and/or two (HIP and HOP) exons in the third cytoplasmic loop (19, 23, 24, 26). Cell-specific expression of PAC1 receptor splice variants determines differential peptide potency and distinct patterns of adenylyl cyclase and phospholipase C stimulation by PACAP38 and PACAP27 (23).

Recent studies have suggested that PACAP peptides may be physiological regulators of sympathetic neuron function. PACAP has been identified in pericellular fiber networks enveloping SCG postganglionic neurons, and sympathetic preganglionic neurons in the spinal cord projecting to the SCG express PACAP (27–29). We demonstrated previously that PACAP peptides are potent and efficacious regulators of SCG neuron transmitter and peptide production, secretion, and mRNA with pharmacological profiles congruous for the PACAP-selective PAC1 receptor (30, 31). Similarly, PACAP peptides selectively and potently stimulate sympathetic neuroblast mitogenesis, neuritogenesis, and survival and sympathetic neuron depolarization and membrane excitability (10, 30, 32).2 Accordingly, PAC1 receptor mRNA has been demonstrated in the rat SCG (30, 31, 33). The cellular targets of PACAP and the mechanisms underlying PACAP sympathetic actions, however, have remained unclear. To further evaluate the mechanisms mediating sympathetic neuron responses to PACAP, the present studies investigated SCG neuron expression of specific PAC1 receptor molecular forms and determined whether PACAP-stimulated receptor activation of specific second messenger pathways modulates peptide secretion. The

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‡ The abbreviations used are: PACAP, pituitary adenylate cyclase-activating polypeptide(s); SCG, superior cervical ganglion; VIP, vasoactive intestinal peptide; NPY, neuropeptide Y; Cy3, indocarbocyanine; PCR, polymerase chain reaction; bp, base pair(s).

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analyses demonstrate the preferential expression of specific PACAP-selective receptor isoforms coupled to multiple intracellular signaling pathways in sympathetic neurons; PAC1 receptor stimulation of phospholipase C is a prominent component of SCG neuron neuropeptide Y (NPY) release.

**EXPERIMENTAL PROCEDURES**

**Animals**—Adult male (225–250 g) and pregnant female Harlan Sprague-Dawley rats were obtained from Charles River Canada; all animal procedures were approved by the University of Vermont IACUC. Animals were decapitated, and SCG were removed and frozen. Adult male (225–250 g) and pregnant female Harlan Sprague-Dawley rats were obtained from Charles River Canada; all animal procedures were approved by the University of Vermont IACUC. Animals were decapitated, and SCG were removed and frozen. Neonatal (postnatal day 1) rat ganglia were obtained from mixed sex litters.

**Cell Culture**—Primary neuronal SCG cultures were prepared as described previously (30, 34). Neonatal rat SCG enzymatically dispersed to produce a pooled population of cells were plated at a density of 1.5 x 10^4 neurons/cm^2, treated with cytosine d-arabinofuranoside to eliminate nonneuronal cells, and maintained in defined complete serum-free medium containing 50 ng/ml nerve growth factor (34). For specific experiments, to obtain neuronal and nonneuronal cell populations containing NPY that coexpressed the PACAP-selective receptor, neurons were incubated with PACAP-selective receptor protein using a modification of the protocol described previously (10, 40). The neurons and sections were incubated for 24 h at 4 °C with 1:2000 affinity-purified rabbit anti-PAC1 receptor antiserum ERIQ, produced against amino terminal extracellular domain of the PAC1 receptor, which did not discriminate among the transcript splice variants. A 449-base pair fragment of the PAC1 receptor cDNA synthesized from rat brain hypothalamus was amplified using the primer templates PACAPR5 and PACAPR6 (Table I). The product was gel-purified and ligated into the EcoRV site of pBluescript II KS+ cloning vector (Stratagene, La Jolla, CA). The nucleotide sequence of the insert was verified by automated fluorescent dye terminator sequencing. Radiolabeled antisense and sense riboprobes were synthesized using [35S]dCTP and T7 or T3 DNA polymerase, respectively; hybridization with sense riboprobes failed to produce detectable signals (data not shown).

**Immunocytochemistry**—Cultured sympathetic neurons on Aclar plastic (39) and cryosections of adult male rat SCG were immunocytochemically stained to localize the PAC1 receptor protein using a modification of the protocol described previously (34, 35, 38). No staining was observed with omission of primary or secondary antisera, incubation with preimmune serum, or absorption of the primary antisemur with immunogen or antigen (data not shown). Immunocytochemically stained samples were viewed by fluorescence microscopy using a Leica DMRB microscope equipped with a Cy3 filter set (Chroma, Brattleboro, VT) or imaged using a Bio-Rad MRC 1000 confocal scanning laser system.

**Table I**

| Primers | Specificities | Sequences | Positions | Annealing temperatures | Product sizes |
|---------|--------------|-----------|-----------|------------------------|--------------|
| PACAPR1 and PACAPR2 | PAC1 receptor (third cytoplasmic loop domain alternative splice variants) | 5′-CCGCTGCTTGAAGTCTCAAGTAGTAACGCGGT5′-CACAGTATTCGCCTTCTCTCC-3′ | 1078–1107 | 60 | 303–471 |
| PACAPR3 and PACAPR4 | PAC1 receptor (amino terminal extracellular domain alternative splice variants) | 5′-AACGACCTGATGGGACCTAAAC-3′ | 211–231 | 56 | 413 |
| PACAPR5 and PACAPR6 | PAC1 receptor (carboxyl terminal intracellular domain; all isoforms) | 5′-CACAGTATTCGCCTTCTCTCC-3′ | 1353–1373 | 56 | 449 |
| VPAC1R3 and VPAC1R4 | VPAC1 receptor | 5′-CGGCAACCGGATCTGGGAAG-3′ | 1034–1054 | 61 | 323 |
| VPAC2R1 and VPAC2R2 | VPAC2 receptor | 5′-AACGACCTGATGGGACCTAAAC-3′ | 1336–1356 | 55 | 396 |

*Positions are given as the nucleotide sequences for the rat cDNA with the following GenBank™ accession numbers: rat PAC 1 (PACAPR5 and PACAPR6), 387 bp, Z23273; PAC1 (short), neither HIP nor HOP1, 303 bp, Z23279; PAC1 (short) (neither HIP nor HOP1), 387 bp, Z23273; PAC1 (short)HOP1, 387 bp, Z23274; PAC1 (short)HOP2, 384 bp, Z23275. The PAC1 receptor amino-terminal extracellular domain very short transcript without exons 4 and 5 and the short variant containing these alternatively spliced exons are predicted to produce 350- and 413-nucleotide amplified products, respectively (44).*
radioimmunoassay using the Biotrak nonacetylation protocol with \(^{125}\text{I}\)-cAMP and Amerlex-M magnetic separation (Amersham Pharmacia Biotech). Assay midpoints were approximately 10 fmol.

**Inositol Phosphate Production—** Total inositol phosphates were quantitated as described previously (42). SCG neurons were cultured in defined medium containing 0.32 \(\mu\)M myo-[\(^{3}\text{H}\)]inositol (19 Ci/mmol, NEN Life Science Products). After 48 h, the cultures were treated with 10 mM LiCl and PACAP38, PACAP27, or VIP. Following phase separation of the methanol/chloroform extract, inositol phosphates in the aqueous phase were separated from free inositol by ion exchange chromatography (AG 1-X8 resin, formate form; Bio-Rad).

**Peptide Level Analysis—** Secreted NPY was evaluated using double antibody radioimmunoassays as described previously (30, 34, 38). Conditioned medium from individual sympathetic neuron cultures (3 \(\times \) 10⁴ cells/well) was assayed using 1:9000 rabbit anti-NPY (RIN7180; Peninsular Laboratories, Belmont, CA) and \(^{125}\text{I}\)-NPY (Amersham Pharma- cia Biotech).

**Messenger RNA Analysis—** Total RNA prepared using RNA STAT-60 total RNA/mRNA isolation reagent (Tel-Test “B”, Friendswood, TX), was used to synthesize first strand cDNA using SuperScript II reverse transcriptase and oligo(dT) primers with the SuperScript Preamplification System (Life Technologies, Inc.) as described previously (29, 30, 35, 37, 38). Amplification with AmpliTaq DNA polymerase (Perkin-Elmer) using the AmpliWax PCR gem-facilitated hot start (30) was conducted using oligonucleotide primers specific for the identification of PAC₁ receptor splice variants in the third cytoplasmic loop or amino-terminal domain or for VPAC₁ or VPAC₂ receptors (Table I). Complementary DNA synthesis in the absence of either RNA or reverse transcriptase or amplification without template, primers, or DNA polymerase failed to yield products (data not shown).

The identities of the amplified products were established by diagnostic restriction endonuclease analysis (43). Verification of HIP or HOP alternative splice variant expression was performed using sequence-specific hybridization as reported previously (38) with the synthetic antisense internal HIP-specific (5’-GTCTGAGGCACAGGGG-GTCTCTCGGGTTTTTTCGT-3’) or HOP-specific (5’-TGACATCTTGCAA- GAGTGCTGCTAGGGCTGTGGCTT-3’) probes end-labeled with \(^{32}\text{P}\)ATP using T4 polynucleotide kinase to equal specific activities. Blots were apposed to autoradiographic film or analyzed by storage phosphor imaging.

**RESULTS**

**Principal Postganglionic Sympathetic Neurons Express PAC₁ Receptor mRNA and Protein—** Neuroanatomical, pharmacological, and electrophysiological studies have implicated PACAP peptides among the physiological regulators of sympathetic neuron function (10, 27–30). The presence of PACAP-selective PAC₁ receptor expression in the cellular elements of the rat SCG was investigated morphologically. Using a riboprobe that did not discriminate the PAC₁ receptor transcript splice variants, hybridization was restricted to SCG principal neurons (Fig. 1A); almost all of the neurons were labeled. PAC₁ receptor immunoreactivity directly paralleled the morphological localization of the receptor transcripts; nearly all of the sympathetic neurons exhibited PAC₁ receptor staining (Fig. 1B). PC12 cells (Fig. 1F), which express PACAP-selective receptor mRNA and respond potently to PACAP, but not VIP (11), also stained, whereas no staining was observed in AtT-20/D16v cells (data not shown), which are regulated by both PACAP and VIP but express VPAC₂ receptor transcripts (35).

The cellular sites of the PAC₁ receptor protein in the post-
ganglionic neurons of the SCG were evaluated using purified primary cultured sympathetic neurons, which exhibited punctate PAC1 receptor immunoreactivity localized predominantly to the soma (Fig. 1C). The cellular distribution of the PACAP-selective receptor protein, examined by fluorescent confocal microscopy, was over the plasma membrane often in patches that may represent receptor clusters (Fig. 1D).

Postganglionic sympathetic SCG neurons are catecholaminergic but are heterogenous with respect to neuropeptide expression. To demonstrate that the subpopulation of SCG neurons synthesizing NPY has the capability to selectively respond to PACAP, the neurons were dually labeled. Approximately 60% of SCG neurons expressed NPY immunoreactivity; all of the NPY-positive neurons coexpressed PAC1 receptor immunoreactivity (Fig. 1E), implicating PACAP among prominent physiological regulators of sympathetic postganglionic neuron NPY expression.

**SCG Neurons Predominantly Express the HOP Isoform of the PAC1 Receptor**—To define the signaling mechanisms underlying PACAP-elicted responses in sympathetic neurons, the molecular forms of PAC1 receptor mRNA were characterized. Multiple PAC1 receptor transcript isoforms that differ in either the amino-terminal extracellular domain and/or the third intracellular cytoplasmic loop were characterized. Alternative splicing in the region encoding the amino-terminal extracellular domain and third cytoplasmic loop generates multiple isoforms of the PACAP-selective PAC1 receptor. The presence or absence of two 84-base pair exons in the region encoding the third cytoplasmic loop produces transcripts with the 22-amino acid residue HIP (light gray) and/or HOP (black) cassettes. Alternative splicing of exons 4 (21 nucleotides) and 5 (42 nucleotides) encodes a 21-residue fragment (dark gray) in the extracellular domain of the receptor, producing either the short or very short isoforms. Data are adapted from Refs. 23 and 44.

Specific hybridization with exon-specific oligonucleotide probes and diagnostic restriction endonuclease analysis. Hybridizations with the HOP-specific oligonucleotide identified one major band, corresponding to the 384/387-nucleotide-amplified product with the HOP cassette insert (Fig. 4A); longer exposures revealed the expression of the much less abundant PAC1 receptor mRNA containing both HIP and HOP exons. Hybridization with the HIP-specific oligonucleotide probe was revealed only after long autoradiogram exposures. Since the two probes were comparable in size, GC content, and specific activity, this suggested that the mRNA expression for the one-cassette PAC1-HIP receptor isoform was much lower than that for the HOP receptor; storage phosphor imaging analyses of the sequence-specific hybridization blots confirmed this relative receptor mRNA expression. Consistent with this finding, the 384/387-nucleotide products were not digested by the HIP exon-specific AvaII restriction enzyme; cleavage with BpI yielded a recognition sequence common to HOP1 and HOP2 digested the PCR product to apparent completion (Fig. 4B), suggesting that virtually all of the PAC1 receptors in the SCG neurons represented the HOP isoform. The HOP1 and HOP2 receptor variants result from alternative usage of two consecutive consensus splice acceptor sites at the 5′-end of the HOP exon, and the presence of the three upstream nucleotides in the HOP1 isoform generates a recognition site for the restriction enzyme PvuII. Digestion with PvuII yielded a predominant 328-nucleotide fragment (Fig. 4B), indicating primary expression of the HOP1 receptor transcript variant; the residual uncleaved material most likely represented the HOP2 isoform. Parallel reverse transcription-PCR of SCG cDNA using primers specific for either HIP or HOP cassettes generated amplified products congruous with the predominant expression of PAC1-HOP receptor transcripts (data not shown).

**SCG Neuronal PAC1 Receptors Represent the Short Variant**—Alternative splicing of both exon 4 (21 nucleotides) and exon 5 (42 nucleotides) produces PAC1 receptor variants with the presence or absence of a 21-amino acid insert in the amino-terminal extracellular domain (26, 44). The short and very short variants were suggested to modulate PACAP27 and PACAP38 PAC1 receptor binding and potency in stimulating phospho-
lipase C activity. Accordingly, SCG neuron expression of PAC1 receptor mRNA amino-terminal extracellular domain splice variants was investigated. Reverse transcription-PCR suggested that the sympathetic neurons expressed predominantly the PAC1(short) receptor transcript variant containing both exons 4 and 5 encoding the 21 amino acid insert in the amino terminus (Fig. 5). Restriction analyses of the 413-nucleotide material using the exon-specific enzymes yielded cleavage patterns expected for the region of the PAC1 receptor cDNA containing the 63-nucleotide insert, whereas the transcript variant in guinea pig SCG contains only exon 5.3 Cleavage with endonucleases with unique recognition sequences in the other exons verified that all of the DNA segments in the fragment were represented (data not shown).

PAC1 Receptor Transcripts Are Preferentially Expressed in SCG Postganglionic Neurons—Pharmacologically, SCG neurons appeared to express predominantly the PACAP-selective receptor rather than either of the VIP/PACAP nonselective receptor subtypes (10, 30).2 In contrast to the PAC1 receptor, amplification of SCG cDNA templates with primers specific for either the VPAC1 or VPAC2 receptors did not reveal significant expression of either receptor mRNA in the intact SCG (Fig. 6A). Amplification with VPAC1 receptor primers, which yielded the predicted product from liver cDNA (data not shown), did not produce amplified fragments from SCG cDNA templates. SCG expression of VPAC2 receptor mRNA was very low compared with that of the PAC1 receptor. To establish the cell type expressing the VPAC2 receptor, purified SCG neuron and nonneuronal cell cultures were prepared by differential plating. The pure postganglionic neurons demonstrated only PAC1 receptor transcript expression, which correlated well with in situ hybridization and immunocytochemical studies (28, 45); the nonneuronal cell cultures, in contrast, appeared to express only VPAC2 receptor mRNA (Fig. 6B).

PACAP27 and PACAP38 Are Potent and Efficacious Activators of SCG Neuronal cAMP and Inositol Phosphate Production—Distinct PAC1 receptor isoforms have been postulated to be differentially coupled to adenyl cyclase and/or phospholipase C (23, 26). Based on previous transfection studies, activation of PAC1(short)HOP1 receptors in SCG neurons by both molecular forms of PACAP were anticipated to elicit nearly equal high potencies in cAMP production, whereas PACAP38 was expected to be orders of magnitude more potent than PACAP27 in stimulating inositol phosphate production. VIP was not expected to potently stimulate intracellular signaling pathways. Unexpectedly, the patterns of SCG neuron expression of either receptor mRNA in the intact SCG (Fig. 6A).
PACAP38 potently stimulated inositol phosphate production in primary cultured SCG neurons (3.0 × 10⁴ neurons/well) and was extracted for cAMP radioimmunoassay. B, sympathetic neurons were incubated in defined medium containing 50 μM RO20-1724 and 10⁻¹¹ to 10⁻⁶ M PACAP27 (●), PACAP38 (○), or VIP (▲) and were extracted for cAMP radioimmunoassay. PACAP27 and PACAP38 potently and efficaciously stimulated sympathetic neuron cAMP and inositol phosphate production. While previous reports suggested that only PACAP38 potently stimulated phospholipase C, both PACAP27 and PACAP38 potently stimulated sympathetic neuron cAMP production. The potency of PACAP38 was approximately 10-fold greater than PACAP27; half-maximal stimulation of cAMP production with PACAP38 was observed at 0.3 nM, and the half-maximal effect of PACAP27 was 3 nM (Fig. 7A). However, PACAP27 appeared more efficacious than PACAP38 in stimulating cAMP production; the maximal level of PACAP27-stimulated SCG cAMP production was approximately 1.4-fold greater than that for PACAP38. As predicted by expression of the PACAP-selective receptor type, VIP was over 1000-fold less potent than the PACAP peptides in adenylyl cyclase activation.

While previous reports suggested that only PACAP38 potently stimulated phospholipase C, both PACAP27 and PACAP38 potently stimulated sympathetic neuron inositol phosphate production in sympathetic neurons with identical half-maximal effects of 0.5 nM peptide (Fig. 7B). Similar to the stimulation of SCG cAMP production, PACAP27 appeared more efficacious than PACAP38 in phospholipase C activation. As expected for PACAP-selective receptor activation of intracellular signaling pathways, micromolar concentrations of VIP were also required to stimulate inositol phosphate production.

Inhibition of Phospholipase C Augments PACAP-stimulated cAMP Production—To study the activation and potential interactions of these two intracellular signaling pathways in response to PACAP receptor activation, the PACAP receptor-mediated stimulation of second messenger production was investigated in the presence of adenylyl cyclase and/or phospholipase C inhibitors. Inhibition of adenylyl cyclase with the aminoethyl-1H-pyrrole-2,5,-dione (U73122) (Calbiochem-Novabiochem; Ref. 46) decreased cAMP production to approximately 60% of PACAP-stimulated levels (p < 0.001; Fig. 8A, black bars). Similar homologous phospholipase C inhibition with the aminosteroid 1-[6-((17β-3-methoxyestra-1,3,5-(10)-triene-17-y)-amino)hexyl]-1H-pyrrole-2,5-dione (U73122) (Calbiochem-Novabiochem) decreased basal and PACAP-stimulated sympathetic neuron inositol phosphate production nearly 50% (p < 0.001; Fig. 8B, dark gray bars), in agreement with inhibitor effects reported in other systems (47–50).

In heterologous inhibitor studies, treatment of the neurons with the adenylyl cyclase inhibitor SQ22536 did not alter either basal or PACAP-stimulated inositol phosphate production (Fig. 8B, black bars); SQ22536, furthermore, did not alter the ability of the phospholipase C inhibitor U73122 to diminish inositol phosphate levels (light gray bars). In contrast, U73122 inhibition of phospholipase C not only failed to attenuate, but potentiated, PACAP peptide-stimulated cAMP production; SCG cAMP levels elicited by PACAP38 and PACAP27 in the presence of U73122 were increased 150% compared with either peptide alone (p < 0.001; Fig. 8A, dark gray bars). These results suggest that SCG PAC1(Short)HOP receptor activation of these two signaling pathways resulted from distinct G-protein interactions, but furthermore, activation of the phosphatidylinositol pathway may be a means of modulating the intracellular rise in SCG cAMP levels following PACAP activation.

Sympathetic Neuron PACAP-stimulated NPY Release Is Reduced by Phospholipase C Inhibition—The contributions of the cAMP and phosphatidylinositol pathways to SCG neuropeptide release were examined under identical conditions to those used for the analysis of PAC receptor coupling to intracellular signaling cascades. Incubation of primary cultured SCG neurons with 100 nM PACAP27 or PACAP38 potently and efficaciously stimulated NPY release approximately 10-fold compared with basal levels (p < 0.001; Fig. 9, open bars). NPY release was increased from 24 fmol of NPY/10⁴ cells in control neurons to 220 fmol of NPY/10⁴ cells and 260 fmol of NPY/10⁴ cells with PACAP27 and PACAP38 treatment, respectively. Basal NPY release was 9-(tetrahydro-2-furyl)adenine (SQ22536) (Calbiochem-Novabiochem; Ref. 46) decreased NPY release approximately 10-fold compared with either peptide alone (p < 0.001; Fig. 9, dark gray bars). These results suggest that SCG PAC1(Short)HOP1 receptor activation of these two signaling pathways resulted from distinct G-protein interactions, but furthermore, activation of the phosphatidylinositol pathway may be a means of modulating the intracellular rise in SCG cAMP levels following PACAP activation.
Sympathetic Neuron PAC1 Receptor Expression and Function

The present studies demonstrate that SCG postganglionic neurons express predominantly the PAC1 (short)HOP1 receptor isoform. Moreover, coupling of this receptor isoform to inositol phosphate production contributes to PACAP-stimulated sympathetic neuron NPY release. Unlike many tissues that express not only multiple PAC1 receptor isoforms, but also VPAC1 and/or VPAC2 receptors (17, 18, 23, 25, 51), our studies demonstrated high SCG sympathetic postganglionic neuron expression of only the PAC1 (short)HOP1 receptor splice variant. In contrast, we identified low levels of VPAC2 receptor mRNA in SCG nonneuronal cells, whereas we and others reported that VPAC1 receptor mRNA is not expressed in the SCG (45). Thus, PACAP and VIP have different cellular targets in the SCG, resulting in potentially important functional differences. PACAP27, PACAP38, and VIP not only modulate neuron functions but also nervous system nonneuronal cell cytokines and growth factors (52–57). The high potencies of the PACAP peptides at both PAC1 and VPAC2 receptors suggest that these peptides may possess regulatory functions on both neuronal and nonneuronal cells in the SCG, while the roles of VIP are predicted to be restricted to nonneuronal cell VPAC2 receptor activation. Under conditions that result in increased sympathetic neuron PACAP and/or VIP expression, such as neuronal injury (58, 59), activation of specific receptor subtypes on SCG neuronal and nonneuronal cells may contribute to the neuronal repair response.

PAC1 receptor activation of both adenylyl cyclase and phospholipase C is characteristic of the group III family of G-protein-coupled receptors (44, 60). Alternative usage of the HIP and/or HOP exons in the region of the PAC1 receptor transcript encoding the third cytoplasmic loop, a region important for receptor-G-protein interaction, has been suggested to determine receptor signaling pathway activation selectivity (23, 26, 33). Furthermore, expression of variants differing in the presence (short) or absence (very short) of exons 4 and 5 in the amino-terminal extracellular domain has been suggested to modulate PACAP27 and PACAP38 binding and discriminate peptide potency in second messenger production (26). The corresponding amino-terminal region in the VPAC1 receptor contains essential amino acids defining the VIP binding domain (61–63). Identification of the PAC1 receptor isoforms expressed by SCG neurons and the physiological coupling of these receptors to intracellular signaling pathways was therefore fundamental to understanding the mechanisms of sympathetic neuron responses to PACAP.

In contrast to most nervous tissues, which express predominantly the PAC1 (short) receptor transcript variant with neither HIP nor HOP exons (23, 33), SCG neurons express predominantly the PAC1 (short)HOP1 receptor isoform. PACAP27 and PACAP38 demonstrated comparable high potency in augmenting cAMP levels in transfected LLC-PK1 cells expressing the PAC1 (short)HOP receptor, but PACAP27 was at least 100-fold less potent than PACAP38 in stimulating inositol phosphate production; moreover, PACAP38 stimulated intracellular inositol phosphate ~40-fold less potently than cAMP (23, 26).

Similar patterns of second messenger activation were demonstrated in adrenal medullary chromaffin or PC12 pheochromocytoma cells (11, 64, 65). By contrast, PACAP-mediated second messenger responses in SCG neurons through the PAC1 (short)HOP1 receptor were unique in that both PACAP27 and PACAP38 exhibited high potency in stimulating cAMP and inositol phosphate production. In accord with the pattern of activation predicted for the HOP variant (23), both peptides potently stimulated cellular adenylyl cyclase; unexpectedly, PACAP38 was ~10-fold more potent than PACAP27. Importantly, in departure from these reports, PACAP27 and PACAP38 demonstrated equal subnanomolar high potencies in augmenting inositol phosphate production in sympathetic neurons. In addition, the concentration dependence of PACAP-induced inositol phosphate production was characteristic of the PAC1 (very short) receptor (26) but was unanticipated for the PAC1 (short) variant expressed by SCG postganglionic neurons. These results implied that alternative splicing of the amino-terminal putative peptide binding domain may not represent the only or major determinant of PACAP27 potency in phospholipase C stimulation and suggested that other factors, such as receptor density or expression of specific cell signaling components, may ultimately dictate the cellular responses (60).

Coupling of one receptor to multiple intracellular signaling cascades poses complex issues related to signal integration, and studies have shown multiple potential points of intersection among different signaling pathways. For example, agonist-stimulated increased IP3 production also has been shown to enhance cAMP formation by a calcium-calmodulin-dependent mechanism (66, 67). Contrary to expectation, inhibition of phospholipase C augmented PACAP-mediated cAMP production. Several phosphodiesterase isoforms exhibit serine/threonine kinase-dependent activities, and decreased protein kinase C activity following phospholipase C inhibition may diminish phosphodiesterase activities, resulting in an apparent elevation in cellular cAMP levels (68, 69). Alternatively, expression of specific adenyl cyclase isoforms by SCG neurons may be important. AC9, prevalent in many neuronal tissues, is inhibited by intracellular calcium (70), and decreased calcium mobilization following phospholipase C inhibition could result in sustained AC9 activity and enhanced cAMP production. The phosphorylation state of adenyl cyclase or PAC1 receptors following phospholipase C
inhibition may also affect total cellular cAMP production. Whether any of these mechanisms underlie the observed interactions between PACAP-mediated second messenger products remains to be investigated.

We demonstrated previously that direct activation of either the cAMP or inositol phosphate signaling pathways stimulates sympathetic neuron transmitter and peptide secretion (34); consequently, PACAP stimulation of PAC(1)(short)HOP1 receptor-mediated neurosecretion (30) was predicted to represent a synergistic response of the two signaling cascades. Inhibition of adenyl cyclase did not affect sympathetic neuron PACAP-mediated NPY release; by contrast, phosphatidylinositol signaling, presumably through inositol 1,4,5-trisphosphate-mediated intracellular calcium release, appeared to represent the predominant means of PACAP-stimulated sympathetic neurosecretion. Similarly, U73122 attenuated tachykinin-stimulated adrenal gland corticosteroid and aldosterone secretion and endothelin-1- and GnRH-induced luteinizing hormone release from gonadotropes (49, 50). PACAP, primarily through PAC1(very short) receptors, also stimulates release of acetylcholine from cardiac ganglia parasympathetic neurons amplifying cardiac ganglia parasympathetic inhibition, but the intracellular signaling mechanisms coupled to these receptor-mediated events are unknown (40, 71). PACAP peptides also appear to be noncholinergic secretagogues of adrenal medullary cell peptides and catecholamines (65, 72, 73). Activation of chromaffin cell PAC1HOP1 receptors stimulates cAMP and inositol phosphate production; however, the contributions of these signaling pathways to release remains unclear (9, 74–77).

In summary, molecular characterization has shown that SCG sympathetic postganglionic neurons express predominately the PAC1(very short)HOP1 receptor isoform. Atypical, PACAP27 and PACAP38 binding to this sympathetic neuron receptor variant results in potent and efficacious stimulation of both adenyl cyclase and phospholipase C activities; activation of the latter appears to be a prominent component to the neuronal secretory response. In the VIP/PACAP family of peptide receptors, these studies demonstrate the specific and preferential actions of PACAP and PAC1 receptors in sympathetic autonomic physiology.

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