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Pituitary Adenylate Cyclase-Activating Polypeptide Type I Receptors Mediate Cyclic AMP-Dependent Enhancement of Neuronal Acetylcholine Sensitivity

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SUMMARY

Nicotinic acetylcholine (ACh) receptors (AChRs) on ciliary ganglion neurons are positively regulated by elevated cAMP levels. Vasoactive intestinal peptide (VIP) can act as a first messenger in the regulation, because application of 1 μM VIP rapidly increases both neuronal cAMP levels and ACh sensitivity. We now report that high affinity receptors for a close VIP relative, pituitary adenylate cyclase-activating polypeptide (PACAP), are present on ciliary ganglion neurons and mediate the cAMP-dependent modulation of AChRs. Consistent with the presence of PACAP type I receptors, binding studies revealed sites on the neurons having ~1000-fold higher affinity for the 38- and 27-amino acid forms of PACAP than for VIP, and cAMP radioimmunoassays demonstrated that PACAP38 and PACAP27 are ~600-fold more potent agonists for mobilizing neuronal cAMP than is VIP. In accord with their higher affinity and potency, PACAP38 and -27 (both at 10 nM) increased neuronal ACh sensitivity by ~50% within 10 min, whereas VIP at the same low concentration was ineffective. The increased ACh sensitivity induced by 10 nM PACAP38 or PACAP27 or 1 μM VIP depends on coincident increases in cAMP levels, because treatment of neurons with adenylate cyclase inhibitors blocked both effects. The findings demonstrate the presence of functional PACAP type I receptors on ciliary ganglion neurons that preferentially recognize PACAP38 and -27 over VIP and act via adenylate cyclase to initiate cAMP-dependent enhancement of AChR function. Finally, we detected PACAP38-like material in ciliary ganglia, suggesting a role for the peptide in modulating neuronal AChRs *in vivo*.

Neuropeptides modulate cell functions via intracellular signaling pathways. Typically, the binding of a neuropeptide to its cell surface receptor leads to altered activity of an effector enzyme such as AC or phosphoinositide-specific phospholipase via an intermediary, membrane-associated G protein (1). In the nervous system, the subsequent change in the activity of PKA or PKC and the accompanying changes in intracellular Ca^{2+} levels can produce widespread and prolonged alterations in the properties of synaptic components (2). In particular, the kinetics and availability of voltage- and ligand-gated ion channels are known to be differentially regulated by activation of PKA and PKC (reviewed in Ref. 3). Our work has focused on regulation of the neuronal nicotinic AChR, a ligand-gated ion channel mediating fast transmission at chemical synapses in the nervous system. Neuronal AChRs are encoded by two types of subunit genes (α2–α8 and β2–β4), resulting in receptors assembled in diverse heteromeric combinations (reviewed in Refs. 4 and 5). Whereas diversity in subunit composition is consistent with the heterogeneity observed in AChR conductance classes, other receptor properties are regulated post-translationally by mechanisms that include the cAMP and IP intracellular signaling pathways. Thus, the efficiency of transmission at neuronal cholinergic synapses depends on a number of AChR properties, including their overall state of regulation.

The cAMP and IP pathways are known to alter neuronal AChR function in different ways (reviewed in Refs. 3 and 5). When phorbol esters are applied to chick sympathetic neurons, mimicking activation of the IP pathway, the rate of AChR desensitization is enhanced, without affecting the peak amplitude of the ACh response (6). SP is a neuropeptide known to activate the IP pathway, and it similarly increases

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ABBREVIATIONS: AC, adenylate cyclase; PACAP, pituitary adenylate cyclase-activating polypeptide; PKA, cAMP-dependent protein kinase; PKC, phospholipid/calcium-dependent protein kinase; IP, inositol phosphate(s); ACh, acetylcholine; AChR, acetylcholine receptor; SP, substance P; AA, arachidonic acid; VIP, vasoactive intestinal peptide; ddA, 2′,5′-dideoxyadenosine; SQ-22356, 9-(tetrahydro-2-furyl)adenine; IP_3, inositol trisphosphate; RIA, radioimmunoassay; IBMX, 3-isobutyl-1-methylxanthine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PLC, phospholipase C; $cAMP_{max}$, net maximal peptide-stimulated cAMP production/ganglion equivalent.

the rate of AChR desensitization in sympathetic (7, 8) and ciliary (7, 9) ganglion neurons, presumably by activating PKC (8). SP may function as an endogenous AChR modulator, because SP-like immunoreactivity is present in both sympathetic (10) and ciliary (11) ganglia. In contrast, elevation of intracellular cAMP in ciliary ganglion neurons, either by bath application of membrane-permeant analogs of cAMP or by intracellular injection of cAMP, enhances neuronal ACh sensitivity without appreciably altering the kinetics of AChR desensitization (12, 13). The increase in ACh sensitivity is independent of protein synthesis and is not accompanied by changes in apparent affinity, single-AChR channel conductance, or activation kinetics, suggesting that cAMP triggers a conversion of nonfunctional AChRs in the neuronal plasma membrane to a functionally available state (12), possibly by cAMP-dependent phosphorylation. The capacity for cAMP-dependent receptor conversion is consistent with earlier studies showing that only a small fraction of surface AChRs on ciliary ganglion neurons are normally functional (13, 14) and that the phosphorylation of one AChR subunit is induced after incubation with cAMP analogs and phosphodiesterase inhibitors (15). Thus, the divergent changes in AChR properties produced by the cAMP and IP pathways could be explained as consequences of phosphorylation by PKA or PKC, respectively, producing an increase in the number of functional receptors for one or a change in receptor desensitization kinetics for the other. In a recent report, AA was found to reduce the ACh sensitivity of ciliary ganglion neurons (16), suggesting that release of AA metabolites by Ca^{2+}-dependent activation of phospholipase A_2 also plays a role in the interplay of signaling pathways regulating neuronal AChR function.

It was recently shown that VIP (1 μM) rapidly enhances the ACh sensitivity of ciliary ganglion neurons (17). The results are consistent with a requirement for cAMP mobilization, because VIP produced a coincident increase in free and total intracellular cAMP levels and because the enhanced ACh sensitivity produced by applications of VIP and a cAMP analog were nonadditive (17). Immunohistochemical studies reveal that a VIP-like peptide is found at nerve terminals (reviewed in Ref. 18), where it is often localized with ACh (19), as occurs in presynaptic terminals in the avian ciliary ganglion (11). The co-localization suggests that a VIP-like peptide may be co-released with ACh (reviewed in Ref. 19) and underscores the potential relevance of VIP-related peptides as neuromodulators during periods of synaptic activity in ciliary ganglia. VIP is a member of a peptide family that also includes glucagon, secretin, and PACAP, with VIP and PACAP representing the most homologous family members (20). PACAP exists in two aminated, biologically active forms, containing 27 and 38 amino acid residues, that were originally isolated from hypothalamic extracts and appear to represent alternatively processed forms of a 176-amino acid precursor protein (21). PACAP27 corresponds to the amino-terminal 27 amino acids of PACAP38. Over its first 28 amino-terminal residues, PACAP38 shows 68% identity with human, rat, and sheep VIP (22) and 75% identity with chicken VIP (23). Recent experiments with PACAP-specific antibodies reveal that both forms of PACAP are widely expressed in the nervous system, at sites in areas including the hypothalamus, posterior pituitary, cerebral cortex, and hippocampus that do not overlap with the distribution of VIP (20, 24, 25). Although PACAP promotes neurite outgrowth in pheochromocytoma PC-12 cells (26) and interleukin production in pituitary cells (27) and has been widely implicated as a neurotransmitter or neuromodulator (reviewed in Ref. 20), a relevance to synaptic transmission has not yet been established.

Recent studies have elucidated two major classes of PACAP receptors. Competition binding experiments reveal that PACAP type I sites prefer PACAP38 and -27 over VIP, whereas type II sites have approximately equal, high affinity for the PACAPs and for VIP and are thought to represent VIP receptors (reviewed in Refs. 28 and 29). Activation of VIP or PACAP receptors is assumed to increase cAMP production via AC-stimulatory G proteins, and the differences in receptor affinity are accompanied by corresponding differences in potency for cAMP mobilization. At type I receptors, the effective concentration for half-maximal stimulation of cAMP production (EC_{50}) is 0.1–6.0 nM for both PACAP38 and -27 and 0.5–1.0 μM for VIP. At PACAP type II receptors (i.e., VIP receptors), EC_{50} values for the PACAPs and for VIP are ~1 nM. Type I sites are further subdivided into type IA sites, which have approximately equal and high affinity for PACAP38 and -27, and type IB sites, which bind PACAP38 with 100-1000-fold higher affinity than PACAP27. In addition to their ability to raise cAMP levels, PACAP38 and -27 mobilize intracellular Ca^{2+} through a G protein-mediated, IP_3-dependent mechanism, thereby implicating a parallel activation of the PLC signaling pathway (30). The tissue distributions of PACAP and VIP receptors are markedly different. PACAP receptors and receptor mRNA transcripts are abundantly expressed in the central nervous system, with little expression in peripheral tissues, whereas VIP receptor and receptor transcripts are expressed in peripheral tissues such as lung, liver, and intestine but are present at much lower levels in brain. These findings indicate that, in the nervous system, PACAP and VIP operate predominantly via PACAP type I receptors. In brain membranes, PACAP type I receptors have an apparent molecular mass of 60 kDa and are associated with a G protein (31). Despite their prevalence in the nervous system, however, little is known about the role of either PACAPs or their receptors in neuronal signaling. We therefore initiated this study to determine the type of PACAP receptors present on ciliary ganglion neurons and to examine their role in regulating a critical component of cholinergic synapses, the neuronal AChR. Our results indicate that PACAP type I receptors represent a major binding component on the neurons and that PACAP38 and -27 preferentially activate the receptors, raising neuronal cAMP levels with 600-fold greater potency than does VIP. We also observed the same general rank order of potency in the ability of the peptides to enhance neuronal ACh sensitivity. A possible relevance for PACAP *in vivo* is also supported by the presence of a PACAP-like peptide in the ganglia.

Materials and Methods

Neuron isolation. Neurons were dissociated from embryonic day 13–14 chick ciliary ganglia by using collagenase A treatment and mechanical trituration procedures described in detail previously (13, 17). Embryonic day 13–14 was chosen because at this developmental age the yield of intact neurons is high (~75%) and neuronal ACh sensitivity is strongly regulated by a cAMP-dependent mechanism

(13). The dissociated neurons were plated on polylysine-coated surfaces at densities of 2 ganglion equivalents (about 8×10^3 cells)/15-mm-diameter glass coverslip or 2–6 ganglion equivalents/16-mm-diameter plastic culture well. Before use in experiments, the neurons were allowed to equilibrate for 2–4 hr at 37° in recording solution (see below) supplemented with heat-inactivated 10% horse serum.

Peptide binding sites. The specificity of peptide binding to ciliary ganglion neurons was assessed with a displacement binding assay (e.g., see Ref. 32). Dissociated neurons were plated at 2–3 ganglia/16-mm well and incubated for 1 hr at 22° in recording solution supplemented with 10% horse serum and containing ^{125}I-PACAP27 (50 pM, 2200 Ci/mmol), with or without the indicated concentrations of competing peptide (PACAP38, PACAP27, VIP, secretin, or glucagon). Competing peptides were added to duplicate wells for each condition, from frozen aqueous stocks (100–200 μM), at dilutions appropriate to maintain final incubation volumes of 250 μl. ^{125}I-PACAP27 was used because it is reported to display a lower degree of nonspecific binding than does ^{125}I-PACAP38 (33). After incubation, neurons were washed four times at 4° in recording solution supplemented with 10% horse serum, to remove unbound ^{125}I-PACAP27. For quantification of remaining bound ^{125}I-PACAP27, the final wash solution was removed by aspiration, cells were scraped in 500 μl of 0.6 N NaOH, and ^{125}I radioactivity was assessed with a Beckmann Gamma 4000 γ counter. In each experiment, nonspecific binding was determined in one set of duplicate wells by including an excess of PACAP27 (1 μM) with the ^{125}I-PACAP27, and the counts were subtracted from total counts in the remaining duplicate wells. Typically, nonspecific binding represented <15% of the total binding observed in the absence of competing peptide. The displacement curves were fit with the Hill equation, $y = [C^n/(K^n + C^n)] \times 100\%$, where y is the percentage of sites that bind ^{125}I-PACAP27 in the presence of the competitor at concentration C, n is the Hill coefficient, and K is the IC_{50}, i.e., the concentration of competing peptide required to inhibit binding of ^{125}I-PACAP27 by 50%.

Accumulation of intracellular cAMP. The ability of PACAP38, PACAP27, VIP, secretin, or glucagon to mobilize cAMP in ciliary ganglion neurons was determined using a commercial ^{125}I-cAMP RIA kit. Neurons were plated at 1–6 ganglion equivalents in duplicate wells for each condition, preincubated for 10 min at 37° in recording solution with 10% horse serum and 100 μM IBMX, and then incubated for an additional 2–60 min at 37° in the same solution with test peptides (PACAP38, PACAP27, VIP, glucagon, and secretin). In some experiments, the neurons were first preincubated in the presence of an AC inhibitor (ddA or SQ-22356) for 30–120 min and then incubated for 15 min with inhibitor in the presence or absence of peptide (see Results). After incubations, the solution was removed by aspiration and cAMP was extracted by treatment of the cells with 200 μl of ice-cold 70% ethanol for 12–16 hr at 4°. For each well, extracted material was transferred to a 1.5-ml centrifuge tube and centrifuged at 13,500 × g for 10 min at 4°, and the supernatant was recovered in a fresh tube. Pellets were washed with 100 μl of 70% ethanol and centrifuged again at 13,500 × g for 10 min at 4°, and the second supernatant was combined with the first. Supernatants were dried by vacuum evaporation for 2–2.5 hr, and the dried material was reconstituted in 50 μl of the RIA buffer. The amount of cAMP in each sample tube was determined according to the manufacturer's instructions, after parallel construction of a standard curve with known amounts of cAMP ranging from 25 to 1600 fmol/tube. The cAMP accumulation curves were fit with the Hill equation (see above), where C is the peptide concentration and K is the EC_{50}, i.e., the concentration of peptide required to produce 50% of maximal cAMP production.

Electrophysiology. Whole-cell currents were measured, using patch-clamp methods, from ciliary ganglion neurons maintained on glass coverslips at room temperature (21–24°), as described previously (13, 17, 34). The control recording solution contained 145.0 mM NaCl, 5.3 mM KCl, 5.4 mM $CaCl_2$, 0.8 mM $MgCl_2$, 5.6 mM glucose, and 5.0 mM HEPES, pH 7.4. The patch pipette solution contained 145.6 mM CsCl, 1.2 mM $CaCl_2$, 2.0 mM EGTA, 15.4 mM glucose, and 5 mM Na-HEPES, pH 7.3. Patch pipettes were pulled from Corning 8161 glass capillary tubing and had tip impedances in recording solution of 1.5–3.0 MΩ. The effects of test peptides and AC inhibitors on ACh sensitivity were examined using the same incubation protocols as described above for stimulating cAMP production, except that IBMX levels were usually kept at 10 μM. After the final incubations at 37°, coverslips were transferred to a recording chamber and maintained in recording solution containing IBMX (with or without test peptides or AC inhibitors) throughout the ACh sensitivity assays, which were performed at room temperature for up to 1 hr.

Whole-cell ACh-activated currents were induced by application of 500 μM ACh (dissolved in recording solution) to individual neuron somata held at −70 mV, with rapid pressure microperfusion at 0.35–0.70 kg/cm^2 (5–10 psi). ACh sensitivity was quantified by transforming the current records to conductance (assuming a reversal potential of −11 mV) and then computing the maximal peak conductance at the start of the ACh application pulse (G_0), as described previously (17, 34). For each cell, G_0 was obtained by extrapolating conductance records to time 0, by fitting the records with two exponential functions that describe the fast and slow processes of AChR desensitization, having time constants T_f and T_s, respectively. The perfusion system we used allowed us to routinely observe fast components of desensitization having time constants of ≥50 msec. To control for differences in neuron soma size (membrane area), each G_0 value was normalized to the membrane capacitance of the cell, which was determined just before the ACh trial. The normalized conductance values ($G_n = G_0/C_m$, in units of nanosiemens/picofarad) thereby provide a measure of the peak ACh response density integrated over the entire functional AChR population on the plasma membrane of the cell (ACh sensitivity). Effects of test peptides on neuronal ACh sensitivity are presented as a percentage change (mean ± standard error) from the mean control G_n determined in parallel in the same experiment, and the statistical significance of differences between treated and control neurons was determined by Student's unpaired t test, at $p < 0.05$.

Identification and localization of PACAP in ciliary ganglia. PACAP levels in embryonic day 13–14 ciliary ganglia were determined by using a RIA kit (Peninsula Laboratories) based on an antiserum for PACAP38 that is specific for PACAP38 over PACAP27 and does not cross-react with mammalian VIP. Peptides were extracted by boiling of bisected ganglia for 20 min in duplicate tubes containing 2 N acetic acid (0.2 ganglion/μl). The ganglia and the acetic acid solution were then transferred to a glass, 200-μl, microtissue grinder (Wheaton, Millville, NJ) and homogenized by 10–15 hand strokes. The homogenates were transferred to 1.5-ml microcentrifuge tubes, stored at 4° for 16–64 hr, and centrifuged (in aliquots of 50–100 μl) at 12,000 × g for 30 min at 4°, and the supernatants were recovered and placed at −80° for 30 min. Frozen supernatant samples were evaporated under vacuum (Speed Vac SC110A) at ≤400 mtorr for 1 hr, and the dried material was reconstituted at a final concentration of 0.5 ganglion equivalents/μl of RIA buffer. The amount of PACAP38-like material in each assay tube was determined according to the manufacturer's instructions, after parallel construction of a standard curve with known amounts of PACAP38 ranging from 1 to 128 pg/tube.

Materials. Fertilized white Leghorn chicken eggs were obtained from Spafas (Norwich, CT). Horse serum and collagenase A were purchased from GIBCO-BRL (Grand Island, NY). Routine laboratory reagents, including ACh, poly-D-lysine hydrobromide, and IBMX, were obtained from Sigma Chemical Co. (St. Louis, MO). Glass coverslips (no. 1, 15-mm diameter) were purchased from Propper Glass Company (Long Island City, NY) and patch pipette tubing (Corning 8161) from Garner Glass Co. (Claremont, CA). Secretin and chicken VIP were obtained from Peninsula Laboratories (Belmont, CA); PACAP38, PACAP27, and glucagon were obtained from Bachem (Torrance, CA). Peptide purity was certified by the manufacturers on the basis of chromatographic properties. ^{125}I-PACAP27

(1.5 mCi, 2200 Ci/mmol) was obtained from DuPont-NEN (Boston, MA), and ^{125}I-cAMP RIA kits were purchased from Amersham (Arlington Heights, IL) and from DuPont-NEN. Results obtained with kits from the two manufacturers were indistinguishable. The ^{125}I-PACAP38 RIA kit was obtained from Peninsula Laboratories. The P-site AC inhibitor ddA was a gift from Dr. Roger A. Johnson (State University of New York, Stony Brook, NY). A second AC inhibitor (SQ-22536) was purchased from Biomol (Plymouth Meeting, PA).

Results

PACAP receptor sites are present on ciliary ganglion neurons. The specificity of peptide binding sites on ciliary ganglion neurons was assessed by examining the ability of PACAP and related peptides to compete with ^{125}I-PACAP27 (Fig. 1; Table 1). PACAP38 and -27 were extremely potent competitors; the concentrations required to displace ^{125}I-PACAP27 binding by 50% (IC_{50}) were ≈1 nM for both. The slightly higher potency of PACAP38 versus PACAP27 in competing with ^{125}I-PACAP27 for binding sites on the neurons (Fig. 1; Table 1) was only apparent, because the mean IC_{50} values for the two peptides (0.5 and 1.1 nM, respectively) were not found to be significantly different ($p > 0.1$). In contrast to PACAP38 or -27, VIP was much less potent in displacing ^{125}I-PACAP27, as indicated by an IC_{50} value close to 1 μM. The Hill slopes (mean ± standard error) obtained using PACAP38 (−1.87 ± 0.58, four experiments) or PACAP27 (−0.65 ± 0.16, three experiments) as competitor with ^{125}I-PACAP27 were significantly different from each other ($p < 0.05$) but did not differ detectably from 1.0 or from the slope obtained using VIP (−1.12 ± 0.81, three experiments) as a competitor ($p > 0.1$). Secretin and glucagon, two other members of the VIP/PACAP family, were unable to detectably compete with ^{125}I-PACAP27 at concentrations of 1.0 nM to 10 μM, although some displacement was apparent

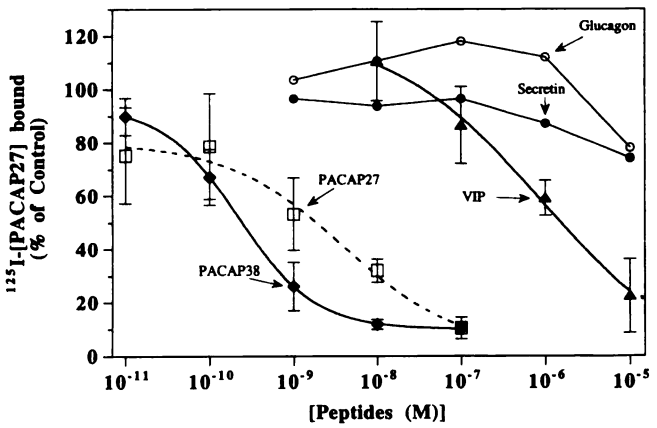

Fig. 1. PACAP receptors on ciliary ganglion neurons show binding specificity. Dissociated neurons were incubated with ^{125}I-PACAP27 in the presence of unlabeled PACAP38 (♦), PACAP27 (□), VIP (▲), glucagon (○), or secretin (●) at the indicated concentrations. For each concentration of peptide, the ability to displace 50 pM ^{125}I-PACAP27 is expressed as a percentage of ^{125}I-PACAP27 bound in the absence of competing peptide. The binding assays were performed in duplicate, and the percentage displacements (mean ± standard error) for three or four experiments using PACAP38, PACAP27, or VIP as competitor are plotted; results are plotted from two experiments using secretin or glucagon as competitor. IC_{50} values were determined as described in the text. The IC_{50} values (mean ± standard error) for each competing peptide are presented in Table 1.

for both peptides at the highest concentration (Fig. 1). These results are consistent with the presence of peptide binding sites on ciliary ganglion neurons that recognize PACAP38 and -27 with high affinity and VIP with ≈1000-fold lower affinity and that do not appear to detectably recognize secretin or glucagon. The findings are in good agreement with the pharmacological profile expected for native PACAP type I receptors (29), previously identified in rat brain membranes (31), and for recombinant PACAP type I receptors expressed in COS Gs1 cells (28).

PACAP receptors mediate stimulation of cAMP production. To determine whether the PACAP binding sites identified above are functional receptors, dissociated ciliary ganglion neurons were incubated with PACAP-related peptides, and the resulting stimulation of cAMP production was measured by RIA (Fig. 2; Table 1). Incubations with the highest tested doses of PACAP38 or VIP for ≥5 min at 37° were sufficient to achieve equilibrium levels of cAMP accumulation (Fig. 2A), and such levels were proportional to the number of dissociated ciliary ganglia used for the assay (Fig. 2B). The $cAMP_{max}$ was obtained by subtracting the basal level of cAMP production/ganglion in the absence of peptide (≈80 ± 7 fmol/ganglion, 39 experiments) from the total amount obtained with a maximal dose. For 10 nM PACAP38 and -27 and 5–10 μM VIP, the mean $cAMP_{max}$ values were similar (≈325 fmol/ganglion) (Table 1). In accord with the displacement binding studies, PACAP38 and -27 were extremely potent in stimulating cAMP accumulation in the neurons (Fig. 2C; Table 1). EC_{50} values, representing the concentrations required for half-maximal cAMP production, were ≈0.5 nM for both PACAPs. In accord with its comparably weak binding activity, however, VIP was 600-fold less potent in stimulating the production of cAMP in the neurons than were either of the PACAPs, having an EC_{50} of about 300 nM. The Hill slopes (mean ± standard error) for cAMP production obtained using PACAP38 (1.56 ± 0.42, six experiments), PACAP27 (3.03 ± 0.82, four experiments), or VIP (0.73 ± 0.12, five experiments) revealed a significant difference from 1.0 only in the case of PACAP27 ($p < 0.05$), suggesting some degree of heterogeneity in the downstream kinetics of cAMP production with the different peptides. Secretin and glucagon failed to detectably stimulate cAMP production in the neurons, even when applied at concentrations as high as 10 μM (Fig. 2C). In other systems, PACAP and VIP stimulate cAMP production by binding to receptors and increasing the activity of AC via an intermediary G protein. The fact that the peptides stimulate cAMP production in ciliary ganglion neurons by activating AC was confirmed with ddA, a specific P-site inhibitor of the enzyme (35). Although preincubation with 200 μM ddA for 1 hr at 37° had no detectable effect on the basal level of cAMP production (data not shown), it reduced $cAMP_{max}$ produced by subsequent treatment with PACAP38, PACAP27, or VIP by ≈70% (Table 1). In three separate experiments, a similar 79 ± 18% reduction in the cAMP accumulation induced by 10 nM PACAP38 was obtained when neurons were pretreated for 30–120 min with 100 μM SQ-22356, which was previously shown to reduce AC activity in mammalian platelets and sympathetic ganglia (36). The ability of forskolin, a direct activator of AC, to increase cAMP production in the neurons was also blocked to a similar degree after preincubation with ddA (data not shown). The binding and cAMP measurement experiments

TABLE 1

Peptide binding to ciliary ganglion neurons and stimulation of cAMP production

Data are presented as means ± standard errors of the number of experiments in parentheses. IC_{50} values represent the concentration of competing PACAP38, PACAP27, or VIP required to displace 50% of bound ^{125}I-PACAP27. The $cAMP_{max}$ values were obtained by subtracting the basal level of cAMP production in control cells from the total amount obtained with a maximal peptide dose (10 nM for PACAP38 and -27 and 5–10 μM for VIP). EC_{50} values represent the peptide concentration required to achieve 50% of $cAMP_{max}$. In experiments with ddA, test neurons were preincubated with inhibitor at 200 μM for 1 hr and then challenged with the indicated peptide at concentrations that maximally stimulated cAMP production; control neurons were not challenged with peptide.

Peptide	Binding, IC_{50}	Stimulation of cAMP production		
		EC_{50}	$cAMP_{max}$	Reduction in $cAMP_{max}$ by peptide + ddA
	nM	*nM*	*fmol/ganglion*	*%*
PACAP38	0.5 ± 0.3 (4)	0.38 ± 0.05 (6)	318 ± 40 (20)	78 ± 9 (6)
PACAP27	1.1 ± 0.6 (3)	0.62 ± 0.12 (4)	369 ± 90 (7)	60 ± 7 (3)
VIP	1028 ± 552[a] (3)	306 ± 42[a] (5)	289 ± 50 (10)	77 ± 2 (4)

[a] Significant difference ($p < 0.05$, by Student's *t* test) from value for PACAP38 or PACAP27.

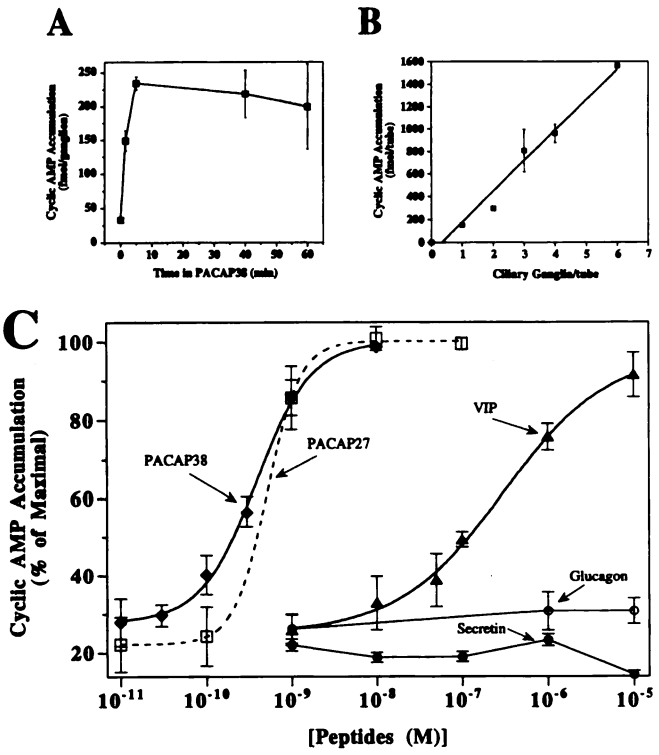

Fig. 2. PACAP receptors on ciliary ganglion neurons are coupled to rapid efficient cAMP accumulation. A, PACAP38 treatment (10 nM) induces maximal cAMP accumulation within 5 min. B, The level of cAMP accumulation is proportional to the number of dissociated ciliary ganglia used in the assay. In A and B cAMP levels were determined as described in the text for increasing times (A) or with increasing numbers of dissociated ganglia per well (B). C, The dose-response relationship for various PACAP-related peptides is shown. Dissociated neurons were incubated for 15 min with the indicated concentrations of PACAP38 (♦), PACAP27 (□), VIP (▲), glucagon (○), or secretin (●). Stimulation of cAMP accumulation was quantified as a percentage of the maximum levels (mean ± standard error), as described in the text. The assays were performed in duplicate, and the average percentages were plotted for four to six experiments in the case of PACAP38, PACAP27, and VIP and for two duplicate-well assays in the case of glucagon and secretin. EC_{50} and $cAMP_{max}$ values were determined for each peptide as described in the text, and the means are presented in Table 1.

indicate that high affinity PACAP type I receptors are present on ciliary ganglion neurons and function to stimulate cAMP production in the neurons via activation of AC.

PACAP enhances neuronal ACh sensitivity. We next compared the ability of PACAP-related peptides to alter the ACh sensitivity of ciliary ganglion neurons. Previous studies showed that ACh at concentrations of 500 μM maximally activated functional AChRs on the neurons and that such responses are enhanced by ~50% within minutes after incubations with 1 μM VIP (17). Because cAMP levels were maximally increased within minutes by 10 nM PACAP38 or -27 but not by 10 nM VIP (Fig. 2C), we compared the ability of the peptides at this low concentration to alter the ACh sensitivity of ciliary ganglion neurons. Whole-cell recordings revealed a rapid increase in conductance after microperfusion with 500 μM ACh (Fig. 3, A and B). Consistent with their high potency in stimulating cAMP production, PACAP38 and -27 applied at 10 nM significantly increased the maximal conductance response normalized per unit of membrane capacitance ($G_n = G_0/C_m$; ACh sensitivity) above levels in paired controls by 53 ± 15% (10 experiments) and 52 ± 23% (10 experiments), respectively (Fig. 3B and C). Such effects are indistinguishable from the 52 ± 11% enhancement in ACh sensitivity seen previously (17) in response to 1 μM VIP ($p > 0.5$, by Student's *t* test). In contrast, VIP at 10 nM, a concentration insufficient to change cAMP levels (Fig. 2), similarly failed to alter neuronal ACh sensitivity. The small 5% reduction in ACh sensitivity for 10 neurons treated with 10 nM VIP was not significantly different from the values for control neurons tested in parallel. For both control and treated neurons, the conductance decayed after the peak because of AChR desensitization (Fig. 3, A and B). In all cases, AChR desensitization kinetics were well described by the sum of fast and slow exponential time constants (Fig. 3, A and B), T_f and T_s, which for 30 control neurons were 0.27 ± 0.02 sec and 3.76 ± 0.30 sec, respectively, with the fast component representing 76 ± 2% of G_0. As in previous experiments using 1 μM VIP (17), these desensitization parameters were not detectably altered by treatment of the neurons with 10 nM PACAP38, PACAP27, or VIP. In all cases, T_f and T_s were about 0.3 sec and 4.0 sec, respectively, and the contribution of the fast component, relative to G_0, was about 75%.

To further examine the mechanism whereby the peptides enhance neuronal ACh sensitivity, we tested their ability to do so after blocking of AC activity. As with the cAMP assays, preincubation with 200 μM ddA had no significant effect on the ACh response observed in the absence of stimulating peptide. The ACh sensitivity of neurons treated with 200 μM ddA was 96 ± 10% (19 experiments) of that obtained in controls not treated with inhibitor, and the kinetics of AChR desensitization were unchanged. When neurons pretreated

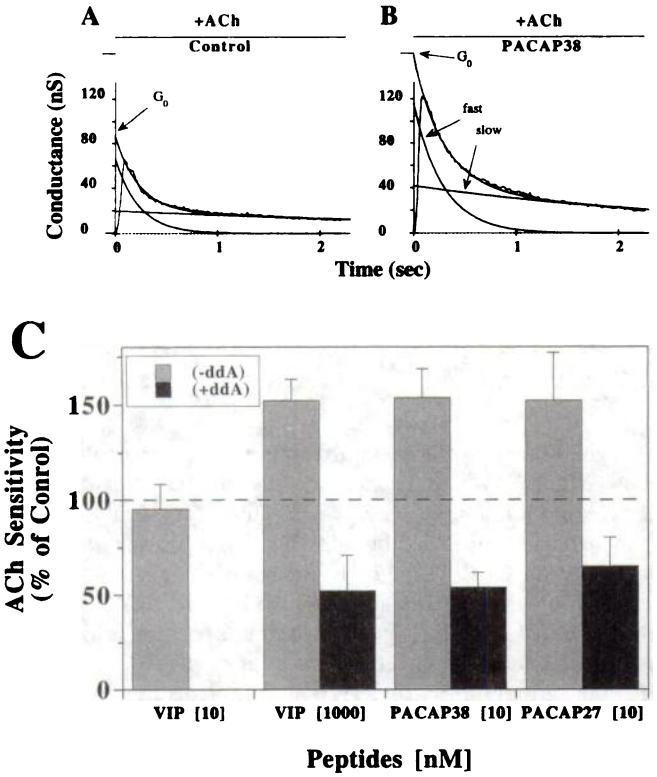

Fig. 3. PACAP38, PACAP27, and VIP increase the ACh sensitivity of ciliary ganglion neurons by a cAMP-dependent mechanism. A and B, Determination of neuronal ACh sensitivity. Neurons were held at −70 mV and exposed to 500 μM ACh by rapid microperfusion (*+ACh*). In control neurons (A) and neurons treated with peptides (e.g., PACAP38 in B), the whole-cell ACh-induced conductance rapidly increased to a peak after the start of perfusion and then decayed in the continued presence of agonist, according to the sum (*solid line fit to data*) of "fast" and "slow" desensitizing components. Note that the maximal peak conductance at the start of the ACh application pulse (*arrow* G_0) was about 2-fold larger for the PACAP38-treated neuron than for the control neuron, whereas the individual desensitization kinetics were similar. Relevant parameters for the neurons depicted in A and B are C_m = 12 and 14 pF, G_0 = 87 and 158 nS, $G_n = G_0/C_m$ = 7.25 and 11.29 nS/pF, T_f = 0.2 and 0.3 sec, and T_s = 4.7 and 3.5 sec, respectively. C, Effects of various peptides on ACh sensitivity (G_n) in the absence (*−ddA*) or presence (*+ddA*) of AC inhibitor. *Bars*, size-normalized maximal conductance values ($G_n = G_0/C_m$) obtained for each cell, expressed as a percentage of control responses in the same experiment. Control neurons were subjected to the same treatment regimen without added peptide. For the ddA experiments, ACh responses of neurons treated with ddA alone (without added peptides) were used as controls. Such values were indistinguishable from those for untreated naive cells tested in the same experiments. Each measurement represents the mean ± standard error of G_n values from 10–55 neurons. Except for 10 nM VIP, all of the treatments revealed a significant difference in mean G_n, compared with control neurons not treated with peptides (*dashed line*). In all cases, neurons were preincubated for 1 hr at 37° in recording solution supplemented with 10% horse serum, without or with the AC inhibitor ddA (200 μM). Test neurons were next incubated for 15 min in the same solution with VIP, PACAP38, or PACAP27 at the indicated concentrations and then examined at room temperature for peak conductance responses to 500 μM ACh, as described for A.

with ddA were subsequently treated with peptide and ddA sufficient to produce a 60–80% block of cAMP mobilization (Fig. 2C; Table 1), the ability of PACAP38 or -27 (both at 10 nM) to increase neuronal ACh sensitivity was abolished (Fig. 3C). In fact, under these conditions, neuronal ACh sensitivity was significantly reduced, to ≈50% of that seen in ddA-treated neurons not challenged with the PACAPs. Similarly, the previously demonstrated ability of 1 μM VIP to enhance ACh sensitivity by ≈50% (17) was also reversed when neurons were preincubated with ddA (Fig. 3C). Consistent with the effects of ddA, preincubation with SQ-22536 also reduced the ACh sensitivity of neurons treated with PACAP38 to 53 ± 7% (four experiments) of that seen for neurons treated with inhibitor but not challenged with the peptide. In all cases, the reduction in ACh sensitivity produced by the peptides after inhibition of AC occurred without an accompanying change in the kinetics of AChR desensitization. The reversal of the ability of the peptides to increase ACh sensitivity when cAMP levels are reduced by inhibition of AC supports the idea that PACAP38, PACAP27, and VIP enhance ACh sensitivity by mobilizing cAMP and suggests that a second pathway, activated in parallel, acts to inhibit AChR function (see Discussion). The electrophysiolgical results demonstrate that PACAP-related peptides enhance the ACh sensitivity of ciliary ganglion neurons with the same order of potency as they increase cAMP levels and that their ability to do so is reversed by inhibition of AC. Viewed with the binding and cAMP production experiments, the results indicate that PACAP38, PACAP27, and VIP bind to PACAP type I receptors on ciliary ganglion neurons and that the coupling of these receptors to increased cAMP production is necessary for enhancing AChR function.

A PACAP-like peptide is present in ciliary ganglia. The presence of a PACAP38-like peptide in ciliary ganglia was determined in ganglion homogenates by RIA using a PACAP38-specific antiserum (Fig. 4). The levels of PACAP38-like material detected by the assay increased linearly with increasing numbers of ciliary ganglia present in the homogenates (Fig. 4A) and averaged 6.7 ± 0.7 pg/ganglion (nine experiments) or 18 ± 2 ng/g of wet weight (Fig. 4B). Compared with other tissues from embryonic day 14 chick embryos, the level of PACAP38-like material in ciliary ganglia was ≈5-fold higher than that in dorsal root ganglia, 10-fold higher than that in liver, and 100-fold higher than that in adrenal gland (Fig. 4B). The amount of PACAP38 immunoreactive material present in ciliary ganglia is comparable to that detected previously (24) in rat cortex (24 ng/g), hippocampus (37 ng/g), and posterior pituitary (40 ng/g) but 6-fold higher than the level in anterior pituitary and 30-fold lower than the level detected in hypothalamus. As a control, the anti-PACAP38 antiserum was tested for its ability to cross-react with VIP, which may be present in the ganglia (11). In two separate experiments, the antiserum was unable to detect exogenous chick VIP when added to the 50-μl assays at concentrations from 1 to 256 pg/tube (data not shown). These findings demonstrate a local source of PACAP38-like material in ciliary ganglia, indicating that a PACAP-like peptide may have functional relevance *in vivo*.

Discussion

The present results can be summarized in three main conclusions. First, chick ciliary ganglion neurons express functional PACAP type I receptors. Second, PACAP type I receptors can mediate the cAMP-dependent enhancement of ACh sensitivity (13, 14) previously demonstrated with high doses of VIP (17). Third, ciliary ganglia contain an endogenous PACAP38-like peptide, indicating that PACAP38 or a

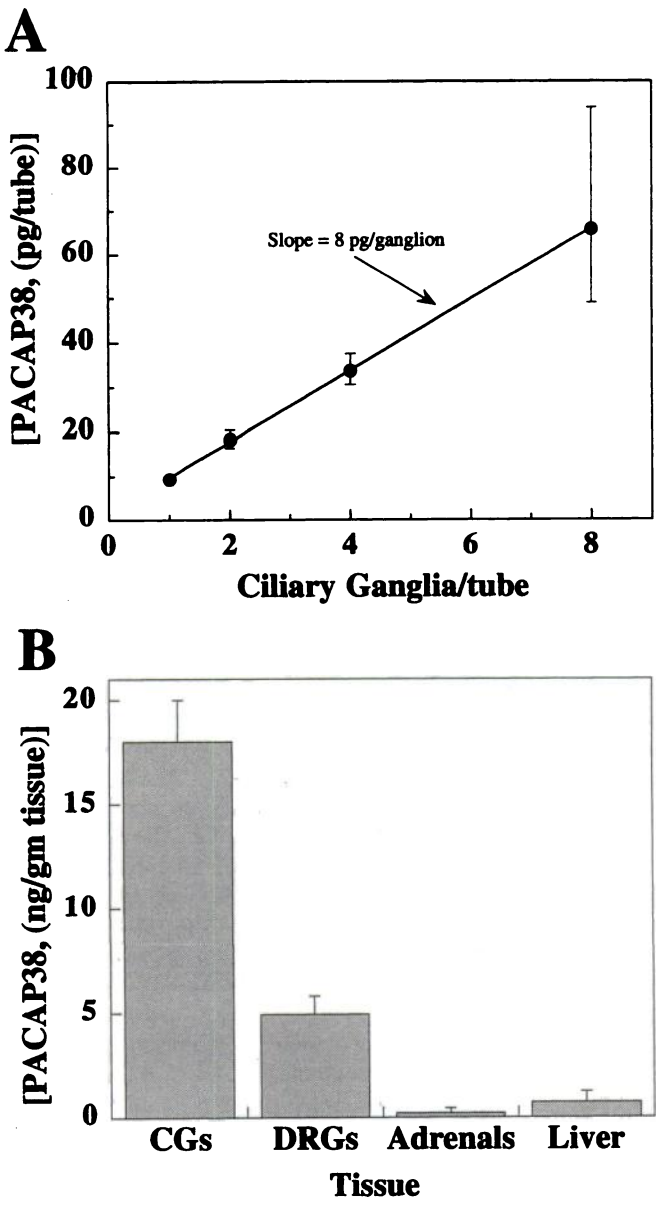

Fig. 4. A PACAP-like peptide is present in ciliary ganglia. A, The amount of material detected is proportional to the number of ganglia. The slope of the relationship provides a measure of the amount of PACAP38-like material/ganglia. B, The distribution of PACAP38-like material in chicken tissues (ng/g of wet weight of tissue) is shown. *CGs*, ciliary ganglia; *DRGs*, dorsal root ganglia.

closely related peptide has relevance in modulating AChR function on the neurons *in vivo*.

We classified the PACAP receptors on ciliary ganglion neurons as type I based on criteria for native PACAP receptors present on brain membranes (31), neuroblastoma cells (37), and PC-12 cells (26), as well as for cloned PACAP receptors expressed on mammalian cell lines (reviewed in Ref. 29). In such cases, as here, PACAP binding sites were characterized by testing the ability of various peptides to displace ^{125}I-PACAP27 and receptor function was assessed by measuring the cAMP produced after peptide application. Of the peptide family that includes VIP, PACAP38, PACAP27, secretin, and glucagon, only the PACAPs and VIP have been found to interact significantly with PACAP receptors. Receptors having nanomolar affinity for PACAP38, compared with micromolar affinity for VIP, and displaying a parallel concentration requirement for cAMP production are classified as type I. Within this class, receptors having approximately equal affinity for PACAP38 and -27 ($IC_{50} \approx 1$ nM) are classified as type IA and those having 100-1000-fold higher affinity for PACAP38 than for PACAP27 are classified as type IB (reviewed in Ref. 29). By the criteria of ligand specificity, binding properties, and relative potencies for cAMP mobilization, ciliary ganglion neurons clearly display PACAP type I receptors. Furthermore, the similar IC_{50} values (~1 nM) obtained using PACAP38 or -27 to compete with ^{125}I-PACAP27 indicate that a significant fraction of the PACAP binding sites can be further classified as type IA. Because the Hill slope obtained using PACAP38 was steeper than that obtained using PACAP27, however, we cannot exclude the possibility that type IB sites having higher affinity for PACAP38 than -27 are also present on the neurons, as was concluded previously for rat brain membranes after examinination of the ability of the same peptides to displace ^{125}I-PACAP38 (38). The lower affinity of VIP in displacing PACAP27 and its lower potency in stimulating cAMP accumulation suggest that type II PACAP receptors (VIP receptors), which are expected to have equally high (~1 nM) affinity for VIP and PACAP (29), either are absent or represent a minor binding component on the neurons. Instead, it seems more likely that the 1 μM concentration of VIP used previously to stimulate cAMP production in the neurons (17) activated the PACAP type I receptors described here.

The ability of PACAP38 and -27 to rapidly increase cAMP production suggests that the PACAP type I receptors present on the neurons activate AC by coupling to G_s. A G protein interaction for PACAP receptors is supported by experiments with solubilized receptors from rat brain membranes, demonstrating that inclusion of guanosine-5′-*O*-(3-thio)triphosphate preferentially reduced the maximal specific binding activity by 70% (31). In addition, cDNAs for PACAP type I and II (VIP) receptors and for secretin and glucagon receptors encode proteins with the seven transmembrane domains characteristic of G protein-coupled receptors. The 35-amino acid region corresponding to the seventh transmembrane domain and the adjacent intracellular region is highly conserved among PACAP type I, PACAP type II (VIP), and secretin receptors, and the intracellular region is considered a potential site for interaction with G_s (28). Regardless of the molecular identity of the regulatory protein, our studies support the idea that PACAP receptors are positively coupled to AC, because inhibition of the enzyme blocked PACAP-stimulated cAMP accumulation in the neurons. PACAP type I receptors and receptor mRNA are expressed at high levels in nervous system regions including the thalamus, hypothalamus, brainstem, spinal cord, hippocampus, and cerebral cortex, with little expression in peripheral tissues (25, 28). In remarkable contrast, VIP receptors and receptor mRNA are highly expressed in lung, intestines, and liver but not in the nervous system (39). These findings, together with the highly restricted cellular localization of PACAP receptor mRNA (28), have led others to speculate that PACAP type I receptors play a role in mediating or modulating synaptic transmission (20, 28). Until now, however, examples of PACAP effects on neuron-like cells have been limited to regulation of neurite outgrowth from adrenal pheochromocytoma PC-12

cells (26) and stimulation of interleukin-6 production in pituitary cells (27). Our results indicate that PACAP, acting through type I receptors, enhances the ACh sensitivity of ciliary ganglion neurons, and they thereby provide the first demonstration that the peptide can act to modulate synaptic transmission in the nervous system.

The evidence that PACAP type I receptor activation increases the ACh sensitivity of ciliary ganglion neurons by a cAMP-dependent mechanism is threefold. First, PACAP38, PACAP27, and VIP all enhanced neuronal ACh sensitivity with the same short latency (10–15 min) and with the same relative potency (PACAP38 = PACAP27 ≫ VIP) as they increased cAMP production. Second, the ≈50% increase in ACh sensitivity produced by 10 nM PACAP38 or -27 was similar to the 50–80% increase previously seen after intracellular injection of cAMP (12) or brief bath application of 8-bromo-cAMP (17). Third, the ability of the peptides to increase neuronal ACh sensitivity was abolished after pharmacological inhibition of AC. Unexpectedly, prior inhibition of AC not only blocked the subsequent ability of VIP, PACAP38, and PACAP27 to increase ACh sensitivity but also reversed the effect, causing the peptides to reduce ACh sensitivity. Pretreatment with AC inhibitors does not result in reduced ACh sensitivity by direct AChR blockade, because the sensitivity of AC-inhibited neurons was indistinguishable from that of untreated controls. Instead, the finding suggests that activation of PACAP type I receptors when AC activity is inhibited unmasks a second signaling pathway that leads to reduced ACh sensitivity. There is substantial evidence from other systems that PACAP type I receptors couple to multiple signaling systems, particularly AC and PLC (reviewed in Ref. 29), and a recent report indicates the presence of five distinct splice variants of the PACAP type I receptor (40) that may differentially alter the pattern of AC and PLC signal transduction. Activation of PLC would be expected to produce IP turnover, culminating in IP_3-dependent mobilization of intracellular Ca^{2+} and activation of PKC (1, 2). Experiments with single rat gonadotrophs indicate that both PACAP38 and -27 stimulate IP_3-dependent Ca^{2+} oscillations (30). If this were the case for ciliary ganglion neurons, the resulting increase in intracellular Ca^{2+} could play an important role in AChR regulation. One mechanism would be through Ca^{2+}-dependent stimulation of phospholipase A_2 (41), causing the release of AA metabolites, because a recent report indicates that AA reduces the ACh sensitivity of ciliary ganglion neurons in a manner that depends on intracellular Ca^{2+} (16). Stimulation of PLC should also lead to activation of PKC. Sustained activation of PKC by phorbol esters does regulate neuronal AChRs, increasing the rate of neuronal AChR desensitization, and SP produces a similar increase in AChR desensitization (7, 9) by a mechanism also thought to involve PKC (8). Under conditions where AC was inhibited, however, we observed that PACAP38, PACAP27, and VIP induced a reduction in ACh sensitivity without a detectable change in the kinetics of AChR desensitization. This difference from previous findings could reflect heterogeneity in the efficiency of different peptide receptor agonists and phorbol esters in activating PKC under conditions where AC is inhibited. Without additional information, however, other explanations, based on the reciprocal cross-talk known to exist between AC and PLC signaling systems (29), are also possible. Taken together, the present findings demonstrate that PACAP type I receptors mediate a cAMP-dependent enhancement of ACh sensitivity that depends on the AC signaling pathway, and they suggest that at least one other signaling pathway is also activated by type I receptors, leading to AChR inhibition. The functional properties of the latter signaling pathway, its relationship to PACAP type I receptors described here, and its interaction with the AC pathway in modulating AChR function still remain to be determined.

Finding evidence for both PACAP type I receptors on chick ciliary ganglion neurons and an endogenous source of a PACAP38-like peptide in the ganglia supports the idea that this peptide/receptor system has relevance for modulating neuronal cholinergic synaptic transmission *in vivo*. The levels of PACAP38-like peptide in ciliary ganglia are comparable to those previously determined in rodent brain (24) and about 5-fold higher than the levels in embryonic day 14 chick dorsal root ganglia, where the neurons do not receive synaptic input. We have not yet determined the cellular localization of PACAP38-like immunoreactivity within the ciliary ganglia, but it seems reasonable to suppose that the cholinergic terminals providing synaptic input to ciliary ganglion neurons would contain PACAP38. Previous experiments indicated that the terminals contacting both ciliary and choroid neurons in pigeon ciliary ganglia displayed VIP-like immunoreactivity (11). It is unlikely that the PACAP38-like peptide in chick ciliary ganglia represents VIP, because a PACAP38-specific antiserum was used and the antiserum did not cross-react with chicken VIP. Given the high degree of amino acid identity between PACAP38 and VIP (23), however, the VIP-specific antiserum used in the earlier study may have cross-reacted with PACAP38. More recent studies with specific antisera indicate nonoverlapping distributions of PACAP- and VIP-like immunoreactivities in the nervous system (20, 24, 25). In addition, the functional type I (PACAP-selective) receptors present on ciliary ganglion neurons are well positioned for activation by PACAP38 released from presynaptic terminals. It is also possible, however, that PACAP38 and VIP are both present in the terminals, with PACAP38 activating type I receptors on postsynaptic neurons and VIP activating type II (VIP) receptors on presynaptic neuron terminals. Future experiments will be directed toward determining whether PACAP and VIP alter cholinergic transmission in ciliary ganglia and how the changes in the ACh sensitivity of ciliary ganglion neurons described here might contribute to the modulation.

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