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VIP Modulates Neuronal Nicotinic Acetylcholine Receptor Function by a Cyclic AMP–Dependent Mechanism

Devorah Gurantz,[1] Alec T. Harootunian,[1,2,a] Roger Y. Tsien,[2] Vincent E. Dionne,[1,b] and Joseph F. Margiotta[3]

[1]Department of Pharmacology and [2]Howard Hughes Medical Institute, University of California at San Diego, La Jolla, California 92093 and [3]Department of Physiology and Biophysics and Fishberg Center for Neurobiology, Mount Sinai School of Medicine, New York, New York 10029

Neuronal nicotinic ACh receptors (AChRs) mediate synaptic transmission throughout the nervous system, and are regulated by cellular processes and interactions that include second messenger signaling pathways. In the case of chick ciliary ganglion neurons, activation of the cAMP-dependent signaling pathway with cAMP analogs enhances ACh sensitivity in a manner consistent with an increase in the number of functional nicotinic receptors. We have now identified vasoactive intestinal peptide (VIP) as a neuromodulator or "first messenger" in the cAMP-mediated pathway that regulates neuronal AChRs. Using cAMP imaging and biochemical detection assays, we find that bath application of VIP elevates intracellular cAMP in freshly isolated ciliary ganglion neurons within minutes. The VIP treatment also enhances neuronal ACh sensitivity assessed with whole-cell recording. The enhanced ACh sensitivity produced by VIP appears with a short latency, similar to that associated with the increase in cAMP, and is not additive with the enhanced ACh sensitivity produced by bath application of a cAMP analog. In contrast, calcitonin gene–related peptide (CGRP), known to regulate muscle nicotinic AChRs via a cAMP-dependent pathway, has no detectable effect on levels of either cAMP or ACh sensitivity in the neurons. The results indicate that VIP enhances the ACh sensitivity of ciliary ganglion neurons via a cAMP-dependent signaling pathway, presumably by interaction with a specific receptor. Since VIP-like immunoreactivity is present in the presynaptic nerve terminals of avian ciliary ganglia, a VIP-like peptide could modulate AChRs *in vivo*.

[Key words: ACh receptor, nicotinic receptor, regulation, ACh receptor function, vasoactive intestinal peptide, electrophysiology, whole-cell recording, patch clamp]

Neuronal nicotinic ACh receptors (AChRs) are ligand-gated ion channels that mediate rapid synaptic transmission in the nervous system. Two types of neuronal AChR subunit genes (α2-α8 and β2-β4) encode proteins that assemble to form oligomeric complexes (reviewed by Schuetze and Role, 1987; Deneris et al., 1991; Role, 1992; Sargent, 1993), possibly in the stoichiometry $2\alpha{:}3\beta$ (Cooper et al., 1991). The efficiency of transmission at neuronal nicotinic synapses will depend on a number of AChR properties including their single-channel conductance and kinetics, as well as their distribution, agonist affinity, desensitization state, and overall ability to function. While the observed heterogeneity in the conductance of neuronal AChR channels can be explained by diversity in subunit composition, other AChR properties are regulated posttranslationally by cellular interactions and mechanisms that include second messenger–mediated signaling pathways.

Second messenger pathways typically culminate in protein phosphorylation, which is known to regulate the properties of both voltage- and ligand-gated ion channels (Levitan, 1988; Levitan and Kaczmarek, 1991), including non-NMDA glutamate receptors (Greengard et al., 1991; Wang et al., 1991) and nicotinic AChRs (Huganir et al., 1986; Miles et al., 1989; Huganir and Greengard, 1990). Both the inositol phosphate (IP) and cAMP signaling pathways have been shown to regulate neuronal AChR function (reviewed by Sargent, 1993; Swope et al., 1993). Using phorbol esters to mimic activation of the IP pathway enhances the desensitization rate of AChRs on chick sympathetic ganglion neurons (Downing and Role, 1987). Substance P (SP), a peptide known to activate the IP pathway, has an effect similar to phorbol esters in sympathetic (Role, 1984; Simmons et al., 1990) and ciliary ganglion neurons (Role, 1984; Margiotta and Berg, 1986). SP may function as an endogenous AChR modulator *in vivo* since SP-like immunoreactivity is present in both sympathetic (Hayashi et al., 1983) and ciliary ganglia (Reiner, 1987). Ciliary ganglion neurons are also regulated by a cAMP-dependent mechanism (Margiotta et al., 1987a; Margiotta and Gurantz, 1989). Treating the neurons with membrane-permeant analogs of cAMP and a phosphodiesterase inhibitor produces a twofold increase in ACh sensitivity within 6 hr, and intracellular application of cAMP causes a >60% enhancement of ACh sensitivity within minutes. The increase in ACh sensitivity is independent of protein synthesis and is not accompanied by detectable changes in either AChR single-channel conductance or kinetics. To account for the increase, it was concluded that cAMP triggers a conversion of nonfunctional AChRs in the neuronal plasma membrane to a functionally available state (Margiotta et al., 1987a), possibly as a result of cAMP-dependent phosphorylation. The cAMP-dependent conversion hypothesis is consistent with earlier studies showing that only a small fraction of surface AChRs on ciliary ganglion neurons is functional under control conditions (Margiotta et al., 1987b; Margiotta and Gurantz, 1989), and that the phosphor-

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Correspondence should be addressed to Devorah Gurantz, Department of Biology 0357, University of California at San Diego, La Jolla, CA 92093.

[a]Present address: NPS Pharmaceuticals, 420 Chipeta Way, Salt Lake City, UT 84108.

[b]Present address: Boston University Marine Program, MBL, Woods Hole, MA 02543.

ylation of one AChR subunit is induced by incubation with cAMP analogs and phosphodiesterase inhibitors (Vijayaraghavan et al., 1990).

In contrast with the ability of cAMP to enhance ACh sensitivity in neurons, elevating cAMP in muscle increases the AChR desensitization rate (Middleton et al., 1988). Calcitonin gene–related peptide (CGRP) is a potent modulator of muscle AChRs that increases both the rate of AChR desensitization (Mulle et al., 1988) and the level of cAMP-dependent AChR phosphorylation (Miles et al., 1989). In addition, CGRP immunoreactivity is present in motoneurons (Fontaine et al., 1986; New and Mudge, 1986) and in motor endplate terminals (Takami et al., 1985; Matteoli et al., 1988), suggesting that the peptide could play a physiological role in regulating muscle AChR function by cAMP-dependent phosphorylation.

Until now, modulators or "first messengers" that regulate neuronal AChRs via the cAMP signaling pathway have not been identified. Vasoactive intestinal peptide (VIP) can modulate neuronal nicotinic transmission presynaptically by enhancing ACh release (Takahashi et al., 1992), and postsynaptically by increasing the excitability of myenteric neurons (Willard, 1990). VIP-like immunoreactivity is observed in nerve terminals distributed throughout the nervous system (Power et al., 1988; Sundler et al., 1988; reviewed by Gozes and Brenneman, 1989), and is often colocalized with ACh (Hockfelt et al., 1980; Lundberg, 1980; Eckenstein and Baughman, 1984; Whittaker, 1989), as occurs in preganglionic terminals of the avian ciliary ganglion (Reiner, 1987). The colocalization with ACh suggests that a VIP-like peptide may act as a cholinergic cotransmitter (Said, 1986; Willard, 1990) since VIP is known to be released from presynaptic terminals by depolarization (Giachetti et al., 1977; reviewed by Whittaker, 1989). In addition, VIP has been shown to increase levels of intracellular cAMP in brain and sympathetic ganglia (Borghi et al., 1979; Volle and Patterson, 1982), presumably by interacting with a specific receptor coupled to a G_s-protein.

Since both VIP and CGRP are present in cholinergic terminals and are thus well positioned to act as modulators, we tested their ability to regulate AChRs on ciliary ganglion neurons via the cAMP-dependent pathway. Using a recently developed cAMP imaging technique (Adams et al., 1991, 1993) we now show that bath application of VIP causes elevation of intracellular cAMP in the neurons within a few minutes. Whole-cell recordings reveal that VIP enhances ACh-induced conductance responses in the neurons with a similar time course, and that the increase in ACh sensitivity produced by VIP is not additive with that produced by application of 8-bromo-cAMP (8Br-cAMP). In contrast with the effects of VIP, bath application of CGRP has no detectable effect on either cAMP or ACh sensitivity in the neurons. The results indicate that VIP enhances neuronal ACh sensitivity via a cAMP-dependent signaling pathway that could be involved in modulating AChRs *in vivo*.

A preliminary account of the findings has appeared (Gurantz et al., 1991).

Materials and Methods

Neuron isolation. Ciliary ganglion neurons were freshly dissociated from embryonic day 13–14 (E13,14) chick embryos using collagenase-A treatment and mechanical trituration procedures described in detail elsewhere (Margiotta and Gurantz, 1989; Gurantz et al., 1993). E13,14 was chosen because at this stage the yield of intact neurons is high (75–80%), and the neurons express large nicotinic ACh responses that are strongly enhanced by a cAMP-dependent mechanism (Margiotta and Gurantz, 1989). Dissociated neurons were plated onto glass or plastic surfaces precoated with poly-D-lysine. Plating densities were two ganglion equivalents (about 8×10^3 cells) per 15-mm-diameter glass coverslip, or 5–15 ganglion equivalents per 35-mm-diameter plastic culture dish. Before any studies were performed, the neurons were incubated at 37°C in recording solution (see below) supplemented with 10% horse serum for 2–4 hr.

Electrophysiology. Whole-cell currents were obtained from ciliary ganglion neurons held at −70 mV using patch-clamp recording methods as previously described (Hamill et al., 1981; Margiotta and Gurantz, 1989). Neurons were examined on glass coverslips at room temperature (21–24°C) using Nomarski optics at 500× magnification. The recording solution contained (in mM) NaCl, 145.0; KCl, 5.3; $CaCl_2$, 5.4; $MgCl_2$, 0.8; glucose, 5.6; and HEPES, 5.0; pH 7.4. For ACh trials, the patch pipette solution contained (in mM) CsCl, 145.6; $CaCl_2$, 1.2; EGTA, 2.0; glucose, 15.4; Na HEPES, 5; pH 7.3. KCl was substituted for CsCl, in cases where direct effects of VIP were assessed. Depending on the particular experiment, stock solutions of chick or porcine VIP, or chick CGRP (all stored at −20°C in 100 μM aqueous aliquots) were added directly to the recording solution to yield the appropriate final concentrations. 8Br-cAMP was added directly to recording solution. Neurons were incubated with the peptides or 8Br-cAMP at 37°C for 10–15 min prior to and then throughout the electrophysiological assay, which was performed at room temperature for up to 1 hr.

Whole-cell currents were induced by applying 0.5 or 1.0 mM ACh dissolved in recording solution to individual neuron somata with pressure microperfusion at 0.35–0.70 kg/cm² (5–10 psi). ACh applied at 0.5 or 1.0 mM was previously shown to activate maximally the AChR population on E13,14 ciliary ganglion neurons (Margiotta and Gurantz, 1989). The ACh-induced currents were recorded using an Axopatch amplifier (Axopatch 1B or 1C, Axon Instruments, Burlingame, CA). The currents were filtered at 5 kHz using the patch amplifier's four-pole Bessel filter, and digitized at 100–250 μsec intervals with a Cheshire data interface (Indec Systems, Inc., Sunnyvale, CA) and laboratory computer equipped with an LSI-11/73 central processor (Digital Equipment Corp., Maynard, MA). The whole-cell conductance for each digitized current value was determined off line as previously described (Gurantz et al., 1993). Since AChRs on the neurons desensitize during prolonged agonist application, ACh sensitivity was quantified from the maximal peak conductance at time $t = 0$ (G_0). For each cell, G_0 was obtained by extrapolating the transformed conductance versus time records to time $t = 0$ by visual fitting with two exponential functions that describe the fast and slow processes of desensitization. To control for differences in neuron membrane area, each G_0 value was normalized to the cell's membrane capacitance (C_m) determined just before the ACh trial. The normalized conductance values ($G_n = G_0/C_m$) thereby provide a measure of the peak ACh response density integrated over the entire functional AChR population on the cell's plasma membrane. For E13,14 ciliary ganglion neurons, the G_n responses were normally distributed, and are expressed here as mean ± standard error of the mean (SEM). ACh sensitivity was also calculated as a percentage ± SEM of the mean control G_n for each experiment. Statistical significance of differences between treated and control neurons was determined by Student's unpaired, two-tailed t test, or for comparisons of more than two conditions, by analysis of variance (ANOVA) using an INSTAT computer program (Graphpad, San Diego, CA).

Fluorescence imaging of unbound cAMP in single neurons. Changes in free intracellular cAMP were monitored with a fluorescent labeled reporter enzyme injected into isolated E13,14 neurons as previously described (Gurantz et al., 1993). The method utilizes recombinant catalytic (C) and regulatory (R) subunits of mammalian protein kinase A (PKA) covalently labeled with fluorescein isothiocyanate and tetramethylrhodamine isothiocyanate, respectively (FlCRhR; Adams et al., 1991, 1993). In the absence of cAMP, the subunits of PKA exist as an R_2C_2 holoenzyme complex that holds the dye moieties sufficiently close together such that excitation of the fluorescein donor results in emission from the rhodamine acceptor by resonance energy transfer. Increased levels of cAMP cause the subunits to dissociate, preventing energy transfer and resulting in an increase in the ratio of emission intensity at two excitation wavelengths: 500–530 nm for fluorescein versus >570 nm for rhodamine. Because the imaging assay involves cAMP binding to an enzyme, the relationship between free [cAMP] and fluorescence emission ratio is expected to be saturable (described in Adams et al., 1993). Calibration of cAMP–FlCRhR interaction can be done in intact cells

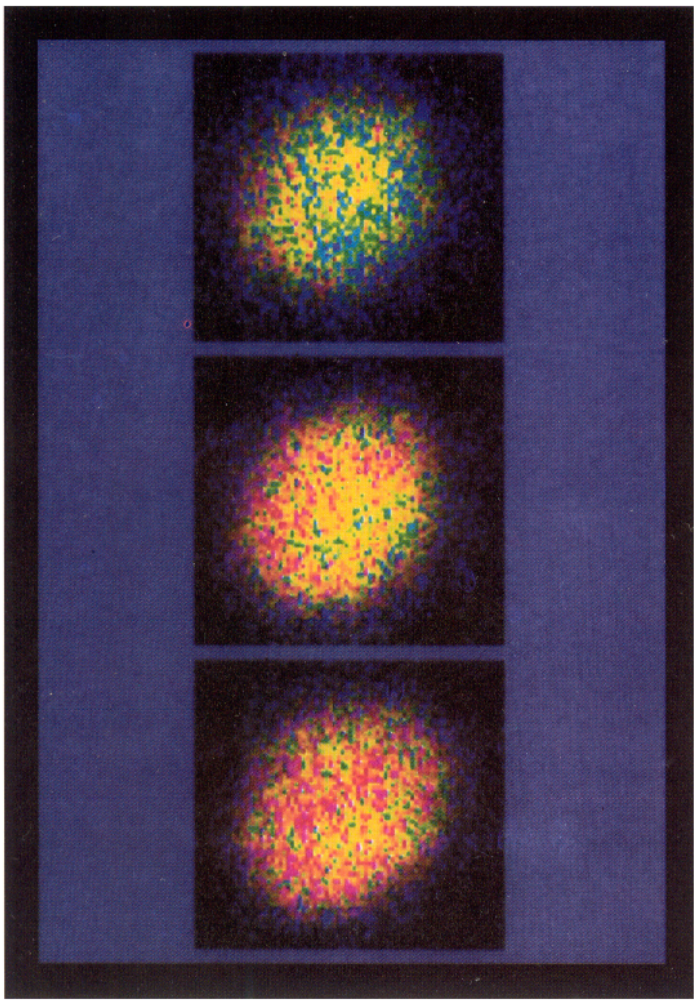

Figure 1. VIP increases levels of free cAMP in individual ciliary ganglion neurons. Ciliary ganglion neurons were injected with the PKA reporter enzyme FlCRhR and examined with fluorescence microscopy. The figure shows pseudocolor images depicting the ratio of fluorescein to rhodamine emission (500–530 nm:570 nm) in a single ciliary ganglion neuron during the control period (*top*), 1 min following the addition of 1 μM chick VIP (*middle*), and following the addition of 1 mM IBMX (*bottom*). The change in color toward the *red* during VIP and IBMX applications indicates an elevation in free intracellular cAMP.

to obtain estimates for cAMP levels (Adams et al., 1993). A full calibration was not performed here. Instead, since the imaging is nondestructive, has good spatial and temporal resolution, and measures free rather than total [cAMP], we used the assay to determine qualitatively if candidate first messenger molecules change intracellular cAMP levels in individual cells, and to estimate the time course of the changes.

Ciliary ganglion neurons from E13,14 chick embryos were isolated as described above and plated in 35 mm culture dishes that were fitted with glass coverslip bottoms precoated with poly-D-lysine. FlCRhR (in 25 mM K_3PO_4, 1 mM EDTA, 0.5 mM β-mercaptoethanol, 2.5% glycerol, pH 7.3) was introduced into cells by manual pressure injection. Final protein concentrations were 0.2–2.0 μM, based on injection of 1–10% of total cell volume. Cells were given at least 30 min to recover following microinjections. A baseline for the fluorescence emission ratio (Adams et al., 1991, 1993) was determined for each cell by repeated measurements for 10 min. Recording solution containing the test agent was then applied, and the fluorescence emission ratio measurements were continued for another 5–30 min. Chick and porcine VIP vary slightly in their amino acid sequence (Mutt and Said, 1974; Nilsson, 1975), but since they did not differ detectably in their effects on ciliary ganglion neurons, results obtained from both forms were pooled. A phosphodiesterase inhibitor (IBMX, 1 mM) was employed as a positive control at the end of each experiment to verify that injected cells could elevate cAMP. The few neurons that failed to display increased cAMP after IBMX treatment were considered damaged and not studied further.

Determining total cellular cAMP. Total cellular cAMP (bound plus free) was determined by the competition between unlabeled, cellular cAMP and a fixed quantity of ^{3}H-labeled cAMP for binding to bovine PKA using an assay kit. Briefly, E13,14 ciliary ganglion neurons were isolated and plated at 5–15 ganglion equivalents/dish on 35 mm culture dishes coated with poly-D-lysine in recording solution supplemented with 10% horse serum. Prior to the assay, the neurons were incubated for 10 min at 37°C with supplemented recording solution alone, with the same solution containing VIP (1 μM), or with forskolin (20 μM), which directly activates adenylyl cyclase and served as a positive control. Following incubation with test agents, the medium was removed and 1 ml of 70% ethanol added to lyse the cells and extract cAMP. The amount of cAMP was determined from tubes containing the 1 ml extract according to the manufacturer's instructions after constructing a standard curve in parallel with known amounts of cAMP ranging from 0.2 to 16.0 pmol/tube.

Materials. Fertilized White Leghorn chicken eggs were obtained from McIntyre Poultry Farms (San Diego, CA) and from Spaffas (Norwich, CT). Horse serum was purchased from Gemini Bio-Products (Calabassas, CA) and collagenase-A from Boehringer Mannheim (Indianapolis, IN). Acetylcholine chloride, poly-D-lysine hydrobromide, and forskolin were obtained from Sigma Chemical Co. (St. Louis, MO). Glass coverslips (#2, 15 mm diameter) were purchased from Propper Glass Company (Long Island City, NY), and glass capillary tubing (Corning #8161), from Garner Glass (Claremont, CA). VIP and CGRP were obtained from Peninsula Laboratories (Belmont, CA). The ^{3}H-based cAMP assay kit (TRK.432) was purchased from Amersham (Arlington Heights, IL).

Results

VIP increases intracellular levels of free cAMP. We used cAMP imaging (Adams et al., 1991, 1993) to monitor the effects of VIP and CGRP application on levels of free intracellular cAMP in individual ciliary ganglion neurons. In 16 of 17 neurons injected with the fluorescent labeled reporter enzyme FlCRhR, subsequent bath application of 1 μM VIP caused a rapid increase in fluorescence ratio, indicative of increased free intracellular cAMP. The signal was maximal after 1–2 min (Figs. 1, 2*A*) and was usually sustained in the presence of VIP for at least 30 min. Similar results were obtained after the application of 2 mM 8Br-cAMP, a membrane-permeant cAMP analog (data not shown). Application of the phosphodiesterase inhibitor IBMX (1–2 mM) also produced a cAMP increase (Figs. 1, 2*A*) that appeared to saturate the reporter enzyme since addition of 2 mM 8Br-cAMP did not increase the fluorescence ratio signal further (not shown). Application of 20 μM forskolin, which activates adenylyl cyclase directly (Seamon and Daly, 1986), also produced increased cAMP levels that were similar in magnitude to those seen with IBMX ($n = 4$ neurons; data not shown). In contrast with the effects of VIP, IBMX, and forskolin, 1 μM chick CGRP did not increase intracellular cAMP levels above baseline. In 12 neurons tested, bath application of CGRP caused no detectable change in the level of free cAMP after 1–10 min, while IBMX induced a substantial increase in the same cells (e.g., Fig. 2*B*).

The ability of VIP to increase the level of free cytoplasmic cAMP in individual ciliary ganglion neurons was confirmed with a competitive binding assay that measures the total cellular level of cAMP (bound plus free) in the neurons. Dissociated neurons were incubated in duplicate dishes at 5–15 ganglion equivalents/dish for 10 min in recording solution supplemented with 10% horse serum, or in the same solution containing VIP (1 μM) or forskolin (20 μM). In three separate experiments, the mean cAMP level was significantly higher by more than twofold ($p < 0.05$) in neurons treated with 1 μM VIP (266 ± 62 fmol/ganglion)

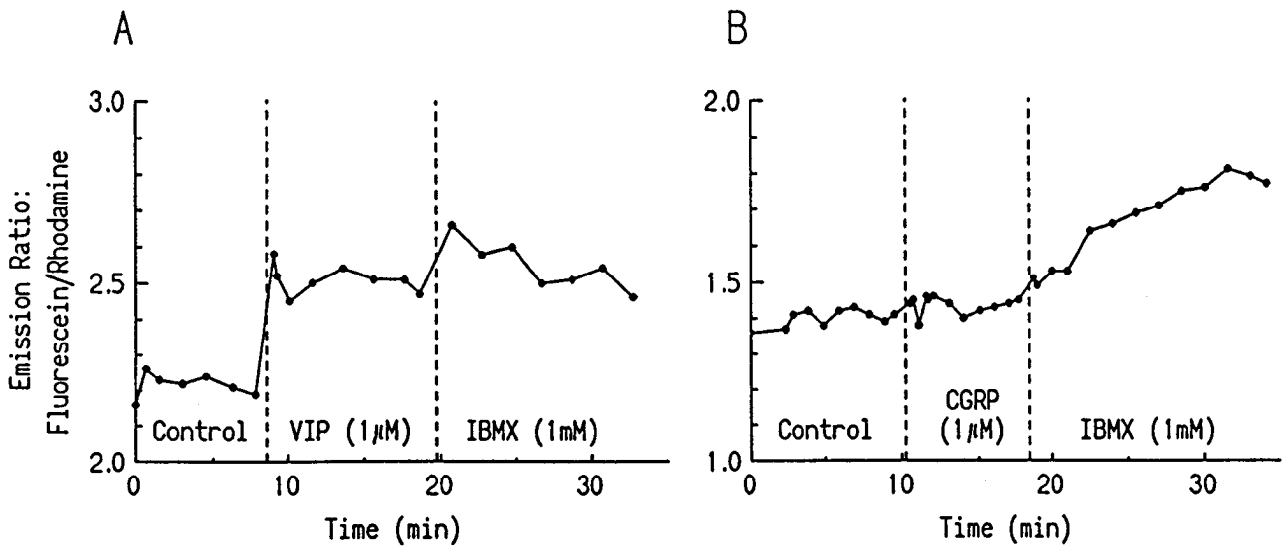

Figure 2. VIP, but not CGRP, rapidly increases free cAMP levels in ciliary ganglion neurons. Experimental results are presented as the emission intensity ratio of fluorescein to rhodamine (500–530 nm:570 nm) for two separate neurons plotted against time. Following a control period, 1 μM chick VIP (*A*) or 1 μM chick CGRP (*B*) was applied. For VIP, the ratio increased in 16 of 17 cells tested (e.g., *A*) indicating an increase in neuronal cAMP concentration. Application of CGRP produced no detectable change of the ratio in 12 cells tested (e.g., *B*). Addition of 1 mM IBMX resulted in increased cAMP levels in the neurons following both types of treatments (*A* and *B*). The ratio measurements were taken at about 1 min intervals, and the data points representing each ratio value were connected. The *dashed vertical lines* separate the different treatments and are drawn at the time of drug addition.

than in untreated controls (125 ± 22 fmol/ganglion). Raising the VIP concentration from 1 μM to 10 μM produced only a 10–20% additional increase in cAMP accumulation (data not shown), suggesting that 1 μM VIP is a near-maximal dose. Using the same assay, 20 μM forskolin also produced a robust increase in total cAMP levels to >4000 fmol/ganglia. Both the imaging and the competition binding experiments reveal that brief exposures to VIP lead to increased levels of cAMP in the neurons. The imaging experiments further indicate that VIP elevates free neuronal cAMP, and suggest that such elevation can account for at least some of the total increase.

ACh sensitivity. We next examined the ability of VIP and CGRP to enhance neuronal ACh sensitivity. Previous studies showed that ACh at concentrations of 0.5–1.0 mM maximally activated functional AChRs on E13,14 ciliary ganglion neurons, and that such responses are enhanced twofold by incubating the neurons with 8Br-cAMP and IBMX for 6 hr (Margiotta and Gurantz, 1989). Since cAMP levels were increased within minutes by VIP (Figs. 1, 2) and 8Br-cAMP but not by CGRP, we compared the ability of 8Br-cAMP, VIP, and CGRP to alter ACh responses of ciliary ganglion neurons following brief incubations. Freshly dissociated E13,14 ciliary ganglion neurons were incubated at 37°C for 10–15 min, either in recording solution alone or in recording solution containing VIP (1 μM), CGRP (1 μM), or 8Br-cAMP (2 mM). The neurons were then tested for responses to ACh in the presence of the same concentration of test agents at room temperature. Consistent with their effects on intracellular cAMP levels, both VIP and 8Br-cAMP significantly increased the peak ACh-induced conductance and the maximal conductance (G_0) following the brief incubations (Fig. 3). In 52 neurons treated with VIP for a total of 10–60 min, the maximal conductance response in VIP-treated neurons normalized per unit membrane capacitance (G_n) was 6.53 ± 0.55 nS/pF, compared with 4.35 ± 0.31 nS/pF for 55 untreated controls ($p < 0.001$). The ability of VIP to enhance the ACh response did not appear to result from a direct change in resting membrane conductance. Application of recording solution containing 1 μM VIP induced only a small outward current of 20–40 pA ($n = 6$ neurons) that coincided with the pressure pulse, and was not detectably different from the current produced by application of recording solution alone. The magnitude of the increase in ACh response produced by VIP was also expressed relative to the mean G_n for untreated control neurons in the same experiment. Using this approach, the mean ACh response was seen to increase by 52 ± 11% after neurons were treated with VIP (Fig. 3*B*, Table 1). A similar enhancement of 80 ± 17% was observed after brief incubations in 8Br-cAMP (Fig. 3*B*, Table 1). The 80% increase in neuronal ACh sensitivity following treatment with 8Br-cAMP is in good agreement with the 66% increase reported previously for ciliary ganglion neu-

Table 1. VIP and 8Br-cAMP enhance neuronal ACh sensitivity

Treatment	# Neurons	ACh response (% of control)
VIP (1 μM)	52	152 ± 11*
Control	55	100 ± 6
8Br-cAMP (2 mM)	39	180 ± 17*
Control	44	100 ± 6
CGRP (1 μM)	15	120 ± 15 (NS)
Control	14	100 ± 18

ACh responses under the indicated conditions are expressed as the mean *percentage* (±SEM) of the normalized conductance response ($G_n = G_0/C_m$) to 0.5 mM ACh (CGRP experiments), or 1.0 mM ACh (all others). Data were compiled for control and treated neurons from 3–10 individual experiments for each of the conditions listed. * Significant difference ($p < 0.001$) between the experimental and control ACh responses; NS indicates no significant difference ($p > 0.05$).

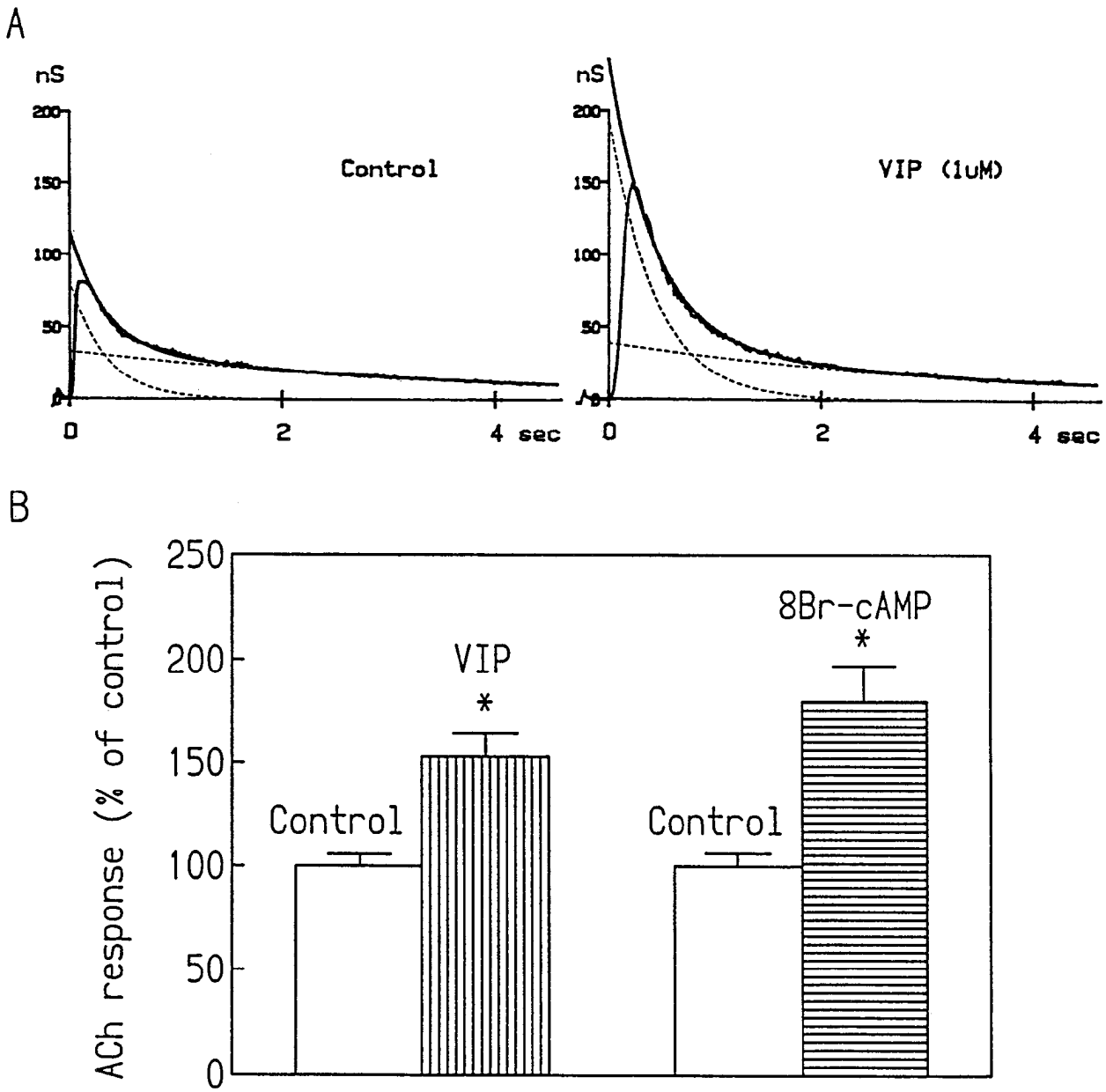

Figure 3. Neuronal ACh responses are enhanced following brief exposure to VIP and 8Br-cAMP. *A*, Neurons were preincubated for 10 min in supplemented saline containing chick VIP (1 μM) at 37°C, and then tested at room temperature for ACh responses in recording solution (also containing 1 μM VIP). The total exposure time to VIP was 10–60 min. Neurons were held in the whole-cell configuration at −70 mV and were challenged with 1 mM ACh by rapid microperfusion at time $t = 0$, for about 4 sec. ACh induced whole-cell conductance responses that are displayed for one control (*left*) and one treated (*right*) neuron from the same experiment. In these and all other cases, the whole-cell conductance response activated rapidly and then decayed in the continued presence of ACh due to receptor desensitization. The *two dashed curves* in each record represent fast and slow exponential components of desensitization, and the *solid curve* fitted to the conductance decay is their sum. G_0 represents the maximal value of the whole-cell conductance obtained by extrapolating the fitted conductance to time $t = 0$ when ACh application began. C_m is membrane capacitance, and G_n represents the quotient G_0/C_m. For the control cell depicted, G_0 = 130.7 nS, C_m = 29.2 pF, and G_n = 4.5 nS/pF; for the VIP-treated cell depicted, G_0 = 262.0 nS, C_m = 22.5 pF, and G_n = 11.6 nS/pF. *B*, Quantitation of ACh responses following brief and sustained exposure to VIP or 8Br-cAMP. Size-normalized conductance values ($G_n = G_0/C_m$) obtained as in *A* above were converted to percentage of control for each experiment. Each value is a mean ± SEM of 39–55 G_n values from neurons obtained from control (*open bars*), VIP (*vertical striped bar*), or 8Br-cAMP (*horizontal striped bar*) groups. Brief treatments (10–60 min) with VIP or 8Br-cAMP enhanced ACh responses by about 50% and 80%, respectively, relative to untreated controls. The *asterisks* indicate $p < 0.001$, by Student's *t* test.

rons in culture after intracellular application of 100 μM cAMP (Margiotta et al., 1987a)

If the enhanced ACh sensitivity produced by a near-maximal dose of VIP (1 μM) is caused by increased intracellular cAMP, it would not be expected to add with that produced when intracellular cAMP is increased by bath exposure to 8Br-cAMP. Whole-cell conductance responses to 1 mM ACh were obtained from control neurons and from neurons treated for 15 min with 1 μM VIP, with 1 mM 8Br-cAMP, or with the two in combination. In the five experiments where additivity was tested, the individual treatments all significantly increased the ACh-induced G_n response above control levels (Fig. 4; $p < 0.005$ for

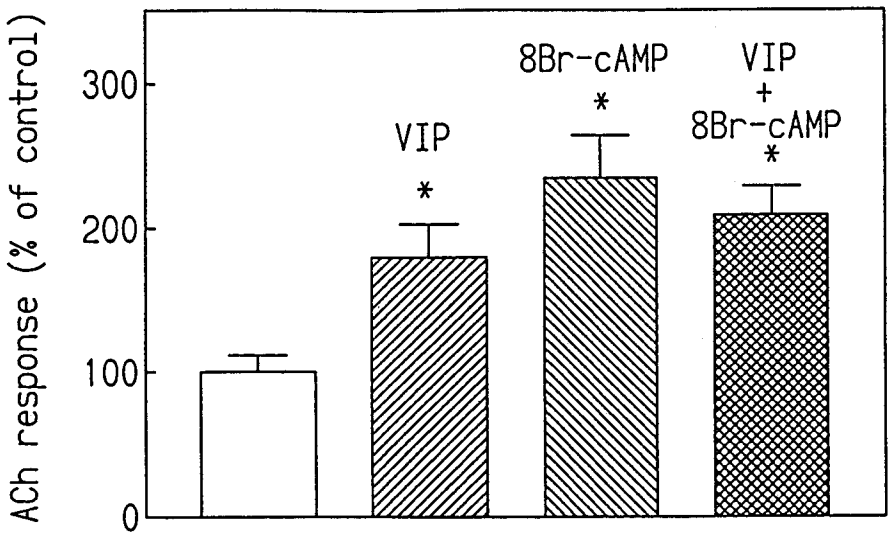

Figure 4. VIP and cAMP effects on ACh sensitivity are not additive. G_n responses were pooled from five experiments, and for each of the indicated treatments are presented as a percentage of control, in a manner similar to that in Figure 3*B*. Neurons were treated with test agents in recording solution for 15 min at 37°C. Whole-cell conductance G_n responses to 1 mM ACh were subsequently assayed at room temperature in the presence of the same concentration of the test agent. Each treatment and control group contained 18–21 neurons. The *asterisks* indicate a significant difference ($p < 0.005$, by Student's t test) relative to untreated controls.

each treatment). There was no significant difference, however, when the enhanced mean ACh response of neurons treated with VIP was compared with that obtained from neurons treated with 8Br-cAMP, or with VIP *plus* 8Br-cAMP (Fig. 4; $p > 0.05$). Since VIP increased the level of intracellular cAMP, and enhanced ACh sensitivity up to but not beyond that seen with 8Br-cAMP, it seems likely that VIP enhances the ACh sensitivity of ciliary ganglion neurons by increasing intracellular cAMP. This conclusion does not exclude the less likely possibility that the VIP and 8Br-cAMP effects converge at a common point in the signaling pathway distal to cAMP.

In accord with the inability of CGRP to increase intracellular levels of free cAMP in the neurons (Fig. 2*B*), short-term exposure to CGRP did not alter neuronal ACh sensitivity. In one of three experiments, CGRP treatment (1 μM, 10–60 min) appeared to enhance the ACh response of five treated neurons over that from four untreated neurons by about 60%. However, subsequent statistical analysis revealed that the apparent difference was not significant ($p > 0.1$, by Student's t test). Data from 15 treated neurons in three experiments revealed no significant difference in the mean G_n values for CGRP-treated neurons and control neurons (Table 1). Others have also noted variability in the capacity of CGRP to modulate the ACh sensitivity of sympathetic neurons (L. Role, personal communication). The VIP, CGRP, and 8Br-cAMP treatments did not change either the fast or slow rates of desensitization, or the relative contributions of the fast and slow desensitization components to the ACh responses of ciliary ganglion neurons. In all cases, the time constants of the fast and slow components of desensitization for both treated and control neurons were 0.3–0.5 sec and 3.5–4.8 sec, respectively, and the contribution of the fast component amplitude relative to G_0 was between 71% and 79%.

Discussion

The present results indicate that VIP can act as a modulator or "first messenger" in the cAMP-dependent signaling cascade involved in regulating nicotinic AChRs on chick ciliary ganglion neurons (Margiotta et al., 1987a; Margiotta and Gurantz, 1989; Vijayaraghavan et al., 1990). Minutes after exposure to VIP, the neurons displayed sustained increases in both intracellular cAMP and ACh sensitivity. The coordinated increases in neuronal cAMP and ACh sensitivity following exposure to VIP suggest that the peptide accomplishes both effects by activating the cAMP-dependent signaling pathway. This conclusion is strengthened by experiments where VIP and 8Br-cAMP were applied, either separately or in combination. The results reveal increased neuronal ACh sensitivity shortly after each reagent was applied separately, but no added enhancement when the two were applied together. Given the relatively small size of the enhancements produced by the individual treatments (50–80%), compared with those seen after longer incubations in 8Br-cAMP (100–200% after 6 hr; Margiotta et al., 1987a), it seems unlikely that the lack of additivity represents a simple ceiling effect at the AChR level. Instead, the results suggest that the individual treatments increase neuronal cAMP sufficiently to saturate initially a downstream component of the signaling pathway, possibly the PKA. It seems likely that the increase in neuronal cAMP results from activation of a VIP-like peptide receptor. A VIP receptor cDNA clone that elevates cAMP in transfected cells has recently been isolated, and VIP receptor mRNA transcripts detected in rat brain (Ishihara et al., 1993). Thus, the simplest conclusion from our observations is that VIP, presumably by acting on its own receptor, modulates neuronal AChR function by rapidly increasing the levels of free intracellular cAMP.

In contrast with the effects of VIP on ciliary ganglion neurons, CGRP, a peptide associated with cAMP-dependent modulation of muscle AChRs but without detectable homology to VIP, produced neither an increase in free cAMP nor a change in the size or kinetics of the neuronal ACh response. Peptide degradation cannot explain the absence of a CGRP effect on the neurons; when applied to myotubes in culture without peptidase inhibitors, CGRP was still able to induce AChR phosphorylation (Miles et al., 1989). The inability of CGRP to alter ACh responses or intracellular levels of cAMP here may indicate that ciliary ganglion neurons lack CGRP receptors coupled to cAMP.

Substantial VIP-induced increases in cAMP were detected within minutes in individual ciliary ganglion neurons using cAMP imaging, and in populations of the neurons using a standard competition binding assay. The imaging experiments indicate that at least some of the increased cAMP freely associates with

the PKA reporter enzyme, FlCRhR. Since cAMP interacts with FlCRhR (Adams et al., 1991, 1993), it is expected that in uninjected cells a portion of the cAMP increase produced by VIP will rapidly associate with native PKA. Previous studies concluded that increasing neuronal cAMP levels acted to convert a portion of a preexisting, nonfunctional AChR pool on ciliary ganglion neurons (Margiotta et al., 1987b) into a functionally available state (Margiotta et al., 1987a), and that such increases also result in phosphorylation of at least one AChR subunit (Vijayaraghavan et al., 1990). While the present results do not link AChR subunit phosphorylation with receptor conversion, they are consistent with such a mechanism since VIP induces a rapid increase in free cAMP that is able to interact with a PKA.

The present experiments indicate coupling between first and second messengers of the cAMP-dependent signaling pathway that regulates neuronal AChRs. AChRs on ciliary ganglion neurons are also sensitive to at least one other modulator, bovine serum albumin (BSA), which produces a threefold increase in ACh sensitivity within minutes of application (Gurantz et al., 1993). Using the same imaging assay, however, BSA did not elevate free cAMP levels in the neurons, suggesting that its ability to modulate AChRs rapidly involves a different form of signal transduction.

VIP may have physiological relevance in the chick ciliary ganglion. A previous study demonstrated VIP-like immunoreactivity colocalized with ACh in presynaptic terminals of pigeon ciliary ganglia (Reiner, 1987). In addition, there is good evidence that VIP and ACh are co-released from presynaptic terminals by depolarizing stimuli (reviewed by Whittaker, 1989), suggesting the peptide normally acts as a cholinergic cotransmitter (e.g., Willard, 1990). The co-release of ACh and VIP from cholinergic terminals in salivary gland and myenteric plexus is approximately equal at the low stimulation frequencies (about 10 Hz; Agoston, 1988; Lundberg, 1981) associated with efficient cholinergic transmission in the chick ciliary ganglion (Landmesser and Pilar, 1972; Dryer and Chiappenelli, 1985). Recent studies indicate that VIP, pituitary adenylate cyclase–activating polypeptide (PACAP), secretin, and glucagon comprise a family of peptides, all positively coupled to adenylyl cyclase and having a high degree of amino acid identity (Arimura, 1992). Because of the similar sequences of the peptides, antisera used in previous immunohistochemical studies may not have been completely specific for VIP. Thus, the identity of the VIP-like peptide actually present in the chick ciliary ganglion still remains to be determined.

VIP displays a broad spectrum of biological activities involved in metabolism, secretion, vascular and muscle tone, cell growth, differentiation, survival, and neuromodulation (reviewed by Said, 1982; Gozes and Brenneman, 1989). Several neuromodulatory roles for VIP are suggested from electrophysiological studies, which reveal both pre- and postsynaptic actions at cholinergic synapses. At neuromuscular (Gold, 1982) and neuronal synapses (Takahashi et al., 1992), VIP enhances nicotinic transmission by increasing ACh release from presynaptic terminals. In addition to the enhancement of postsynaptic AChR function seen here, it is possible that a VIP-like peptide present in the ciliary ganglion also acts presynaptically to modulate ACh release. Such an effect would not have been detected in the present experiments, since presynaptic terminals are removed when the neurons are dissociated. In contrast with the 50% enhancement of ACh sensitivity seen here for chick parasympathetic neurons, no increase in nicotinic response size was detected following VIP application to the frog motor endplate (Gold, 1982), or to mammalian sympathetic neurons (Mo and Dun, 1984; Takahashi et al., 1992). VIP would not have been expected to enhance motor endplate ACh sensitivity, however, since different cAMP-dependent regulatory mechanisms are known to affect AChRs on muscle fibers and neurons (Margiotta et al., 1987a; Middleton et al., 1988; Miles et al., 1989). The inability of VIP to produce a detectable change in nicotinic response from the AChR population on mammalian sympathetic neurons (Mo and Dun, 1984; Takahashi et al., 1992) may have resulted from the different methods used, or may reflect true differences in the regulation of AChR subtypes expressed in sympathetic and parasympathetic ganglia of mammals and avians (reviewed by Sargent, 1993). Previously reported postsynaptic actions of VIP include increased sensitivity of muscarinic receptors in sympathetic ganglia (Mo and Dun, 1984) and increased excitability of postsynaptic neurons at nicotinic synapses in myenteric ganglia (Willard, 1990). The relevance of such postsynaptic effects to the present results seems doubtful, however, since VIP did not detectably alter the holding current in ciliary ganglion neurons, and since ACh responses from the neurons were unaffected by the muscarinic receptor antagonist atropine (Margiotta and Gurantz, 1989). Based on the available data, it seems reasonable to conclude that the modulation of nicotinic AChR function observed here represents a distinct biological activity of VIP, or a related peptide. Such AChR modulation may involve a cAMP-dependent increase in the availability of functional AChRs (Margiotta et al., 1987a). Since neuronal AChRs are distributed at both nonsynaptic and synaptic sites, a VIP-like peptide may serve to regulate the AChR population on ciliary ganglion neurons, and thereby have relevance over periods that include and extend beyond episodes of synaptic activity.

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