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Substance P modulation of acetylcholine-induced currents in embryonic chicken sympathetic and ciliary ganglion neurons

(peptide modulation/acetylcholine receptor desensitization/ganglionic receptors)

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ABSTRACT **Substance P has been identified by combined immunohistochemical and radioimmunological techniques to be present in preganglionic cholinergic and sensory nerve fibers of amphibian, mammalian, and avian autonomic ganglia. The peptide has been shown to depolarize sympathetic neurons of frog and guinea pig and to decrease the cholinergic activation of Na^+ influx and catecholamine release from chromaffin cells. The aim of this study was to examine the interaction of acetylcholine and substance P on autonomic neurons. This report demonstrates a direct effect of substance P on acetylcholine-induced inward currents in both sympathetic and parasympathetic neurons clamped near resting membrane potential. Under these conditions, substance P dramatically enhances the rate of decay of the inward current in the continued presence of agonist without substantially affecting peak inward current. This effect is consistent with an enhancement of acetylcholine-receptor desensitization. Since substance P-containing cell bodies have been demonstrated in the avian (preganglionic) column of Terni as well as in fibers from the nucleus of Edinger-Westphal, the observed peptide inhibition of cholinergic activation of the neurons may function physiologically to modulate synaptic function in autonomic ganglia.**

Over the last 10 years, more than 30 peptides have been found in mammalian nervous tissue. The further demonstration that many of these peptides coexist with more "classical" neurotransmitters underscores the enormous potential for diversity of chemical signaling in the nervous system. The functional roles so far elucidated for various peptides are quite diverse. Perhaps most intriguing, however, in light of peptide–transmitter coexistence, are the modulatory actions of peptides on the effects of classical neurotransmitters. Among these are potentiation by vasoactive intestinal polypeptide of acetylcholine (AcCho)-induced secretion from acinar cells (1), inhibition by substance P of AcCho activation of the Renshaw cell (2) and the chromaffin cell (3–5), and potentiation by substance P and somatostatin of β-adrenergic activation of astrocytes (6).

In the avian nervous system, immunohistochemical techniques have demonstrated substance P in preganglionic cell bodies within both the column of Terni and the nerve fibers from the Edinger-Westphal nucleus (7–9). In the ciliary ganglion, substance P-containing terminals from these preganglionic neurons contact most of the postganglionic cells (7, 8). The presence of substance P in both sympathetic and parasympathetic ganglia of many species (7, 8, 10–13) suggests a possible physiological role for this peptide in ganglionic transmission. Furthermore, the coexistence of substance P and AcCho in preganglionic fibers raises the possibility that the role of the peptide might be to modulate AcCho action. Substance P modulation of AcCho effects in autonomic ganglia have not been examined (but cf. ref. 14), although several studies have demonstrated a direct depolarization of mammalian and amphibian sympathetic neurons by the peptide (15–18).

This paper reports that substance P selectively modulates AcCho-induced depolarization and inward current in chicken sympathetic and parasympathetic neurons. The observed increase in the rate of decay of the AcCho-induced current by substance P occurs immediately and in the absence of substantial effects on peak current. Furthermore, substance P has no direct effect on membrane potential or resting membrane resistance. These results are consistent with an enhancement of agonist-induced desensitization of the receptor, as suggested from previous ion flux studies in chromaffin cells (5), and may underlie the substance P modulation of AcCho-induced release of catecholamine (3, 4). The observed effect of substance P on the rate of AcCho-induced current decay may serve to modulate the cholinergic synaptic input to the neurons *in vivo*. In addition, the substance P modulation of AcCho-induced currents may be a property unique to the ganglionic receptor, because the time course of AcCho-induced currents in embryonic chicken myotubes is not altered by the peptide.

METHODS AND MATERIALS

Culture. Chicken sympathetic neurons were prepared by a modification of previously described techniques (19). Paravertebral ganglia were dissected from 9- to 11-day-old chicken embryos, teased gently to small fragments, and incubated for 20 min at 37°C with 0.01% trypsin (Sigma) in a Ca^{2+}- and Mg^{2+}-free phosphate buffer with 6 mM glucose. The ganglia were then mechanically dissociated to single cells by repeated passage through a fire-polished Pasteur pipette and plated in Eagle's minimal essential medium supplemented with 10% heat-inactivated horse serum/5% chicken embryo extract/penicillin (500 μg/ml)/streptomycin (50 μg/ml)/2.4 mM glutamine/2.5S nerve growth factor (0.5 μg/ml) (gift of E. Johnson, Pharmacology Department, Washington University School of Medicine). The neurons were plated at 10^5 per dish in polyornithine-coated 35-mm plastic dishes (20). Non-neuronal cell growth was not promoted by these growth conditions; the cultures were essentially devoid of background cells.

Chicken ciliary ganglion neurons and myotubes were prepared and plated as described (21, 22) on collagen-coated plastic or glass coverslips in growth medium lacking nerve growth factor. The neurons were studied 2–7 days after plating; muscle was studied 6–7 days after plating.

Electrophysiology. Experiments were carried out at room temperature on the stage of an inverted microscope equipped with phase-contrast optics as described (22). All recordings used the whole-cell patch-clamp technique (23). Recording

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Abbreviation: AcCho, acetylcholine.

medium contained 120 mM NaCl/5.4 mM KCl/1.2 mM NaH_2PO_4/0.8 mM $MgSO_4$/3.6 mM $CaCl_2$/6 mM glucose/12 mM Hepes, pH 7.4. Patch electrodes were prepared from boroscillicate glass (WPI Instruments, Waltham, MA; 1B12OF) by a two-stage pull on a vertical electrode puller (Kopf). The electrodes were filled with a filtered solution containing 140 mM KCl/2 mM $MgCl_2$/11 mM K_2EGTA/1 mM $CaCl_2$/10 mM Hepes, pH 7.3. Signals were monitored with a Dagan 8900 patch-clamp amplifier in both voltage and current clamp mode, using a 10-gΩ or 1-gΩ feedback resistor headstage. The approximate frequency response of these headstages during whole-cell recording is ≈3 kHz. Judging from the lack of notches or indication of aborted spikes on the rising phase of the current records, the cells were adequately space-clamped. Intracellular voltage and current recordings either were displayed on a storage oscilloscope and chart recorder or were recorded for later analysis on FM tape.

Drug Application. AcCho (Sigma) and/or substance P (Sigma or Peninsula Laboratories) were prepared at known concentrations in (filtered) recording medium and applied by pressure ejection from a 2- to 5-μm tip diameter pipette placed 25–50 μm from the cell soma (24).

RESULTS

Using whole-cell current-clamp recording techniques, sympathetic neurons were found to have resting potentials (V_m) of −50 to −70 mV (−58 ± 2, n = 10) and input resistances (R_{in}) of 0.4–1 GΩ. Recordings from ciliary ganglion neurons yielded similar results; V_m = 55.6 ± 1.0 (n = 33) and R_{in} = 0.93 ± 0.08 (n = 27).

Since sympathetic and ciliary ganglion neurons *in vivo* receive peptidergic as well as cholinergic input, the interaction of AcCho and substance P was examined. Application of 10 or 100 μM AcCho to sympathetic neurons caused a rapid depolarization of the cell membrane potential typically accompanied by one or more spikes on the initial rise (Fig. 1*A*). Continued application of AcCho caused a prolonged depolarization that slowly returned toward rest potential, even with maintained exposure to agonist. The concurrent application of AcCho and substance P (10 or 20 μM) produced a very different response; the membrane repolarized to rest potential rapidly (within 2 sec) in spite of the continued presence of AcCho (Fig. 1*B*).

To determine whether the effect of substance P on AcCho-induced depolarization was due to a direct effect of the peptide on resting membrane potential or input resistance, the effect of substance P alone was examined. Fig. 1*C* is a whole-cell current-clamp recording in which the membrane potential ($V_m \approx -60$ mV) was monitored and changes in R_{in} were measured by injection of constant current pulses through the recording electrode. Application of substance P (20 μM) had no effect on either V_m or R_{in}. In addition, substance P had no effect on the amplitude or duration of the somatic action potential evoked by direct stimulation of either sympathetic or ciliary ganglion neurons (data not shown).

The above experiments demonstrate that substance P can inhibit AcCho-induced depolarization without changing resting membrane conductance or by altering the voltage-dependent channels underlying the action potential. To test for a direct effect of substance P on the AcCho-induced currents, the neurons were voltage-clamped at resting membrane potential (−60 mV), and current flow in response to AcCho in the presence or absence of substance P was measured. Any voltage-dependent conductances that might be activated by the AcCho-induced depolarization in unclamped neurons would not be activated in these cells clamped at rest.

Fig. 2 *A1* and *B1* illustrate typical responses of voltage-clamped sympathetic neurons to a 10-sec application of 10 and 100 μM AcCho, respectively. Inward current increased rapidly to an initial peak value (I_{peak}), with a large increase in the current noise, and then, in the continued presence of AcCho, it sagged back toward baseline. This decay in the response results in an inward current level 5 sec after the onset of AcCho application (I_5) that is 10% or 25% (for 10 and 100 μM AcCho, respectively) lower than I_{peak}. The rate of relaxation of inward current in the continued presence of agonist (which can be measured by the I_5/I_{peak} ratio) is therefore dependent on AcCho concentration (compare Fig. 2*A1* with Fig. 2*B1* and Table 1) and is probably analogous to the agonist-dependent desensitization described at the nerve-muscle synapse (24–28).

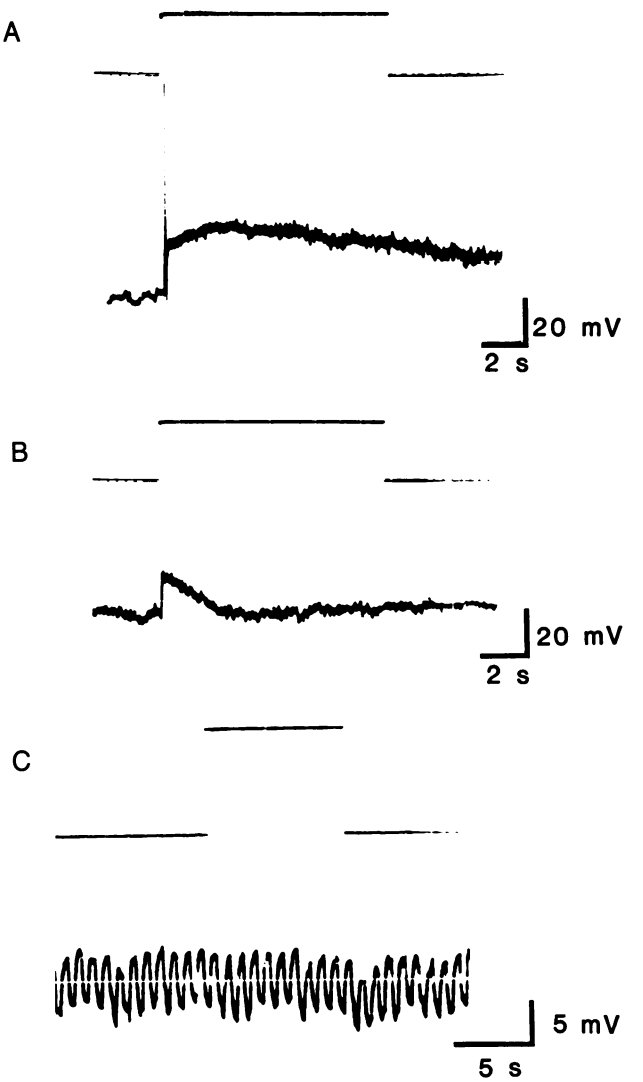

FIG. 1. Substance P inhibits AcCho-induced depolarization but does not affect resting membrane potential or resistance. Whole-cell current clamp recording from sympathetic neuron 5 days *in vitro*. V_m = −58 mV. Upper trace in each set of records indicates the duration of drug application from a nearby pressure ejection pipette. (*A*) Response to 10-sec application of AcCho (100 μM). (*B*) Response of the same neuron to concurrent application of AcCho (100 μM) and substance P (20 μM) from a second pressure-ejection pipette. All other conditions are identical to *A*. Note rapid repolarization of the neuron in spite of continuous application of AcCho. (*C*) Whole-cell current clamp recording from sympathetic neuron 5 days *in vitro* (V_m = −58 to −61 mV). Constant current pulses of 350-msec duration (sufficient to achieve steady-state ΔV) were injected through the bridge circuit of the recording device for measurement of input resistance. Substance P (20 μM) has no effect on membrane potential or input resistance.

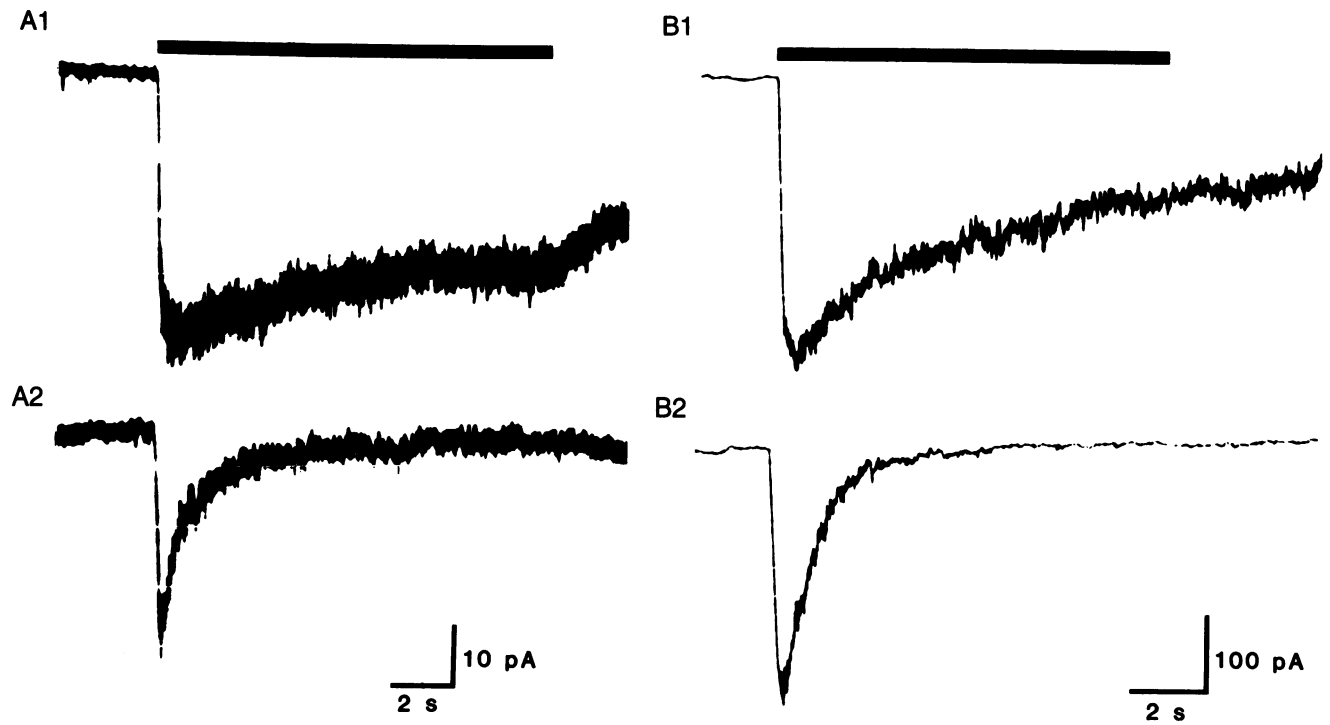

FIG. 2. Substance P increases the rate of decay of AcCho-induced currents in sympathetic neurons. Voltage-clamp records from two neurons (A and B) clamped at rest potential (−60 mV). AcCho alone or AcCho and substance P were applied at the following concentrations for the time indicated by the bar (≈10 sec): (*A1*) 10 μM AcCho; (*A2*) 10 μM AcCho and 10 μM substance P; (*B1*) 100 μM AcCho; (*B2*) 100 μM AcCho and 20 μM substance P. Note the rapid decline in AcCho-induced inward current with substance P.

Concurrent application of substance P with AcCho to sympathetic neurons clamped at rest potential had little or no effect on peak inward current. Furthermore, if substance P alone was applied before (<1 to 5 sec; 10-sec pulse) the concurrent application of AcCho and substance P, the peak current was only slightly decreased compared with control. I_{peak} with prior application of substance P was 0.80 ± 0.05 (n = 16) of control, including tests of the recovery of the AcCho and substance P response. However, substance P markedly decreased the duration of the net inward current and diminished the AcCho-induced current noise in the continued presence of agonist (Fig. 2 *A2* and *B2*). This resulted in a dramatic increase in the rate of decay of the inward current. Thus, I_5/I_{peak} in the presence of AcCho alone was 5–25 times greater than I_5/I_{peak} with AcCho and substance P. This effect was seen in all sympathetic neurons tested (n = 37). The results obtained with both 10 and 100 μM AcCho in the presence and absence of substance P are summarized in Table 1. Substance P affected the rate of decay of nicotine-induced currents in a quantitatively similar manner and had no effect on muscarine-induced inward currents in a few neurons in which this muscarinic agonist was effective (data not shown). Substance P, therefore, has a direct effect on the AcCho-induced currents mediated by nicotinic receptors. This result is consistent with the selective inhibition of nicotinic-stimulated catecholamine release from chromaffin cells by substance P (3, 4).

Table 1. Inward current decay (I_5/I_{peak})

	Sympathetic neurons		Ciliary ganglion neurons	Muscle
	100 μM AcCho	10 μM AcCho	10 μM AcCho	10 μM AcCho
AcCho alone	0.72 ± 0.03 (10)	0.92 ± 0.04 (5)	0.82 ± 0.05 (4)	0.98 ± 0.01 (4)
AcCho and SP	0.13 ± 0.03 (10)	0.17 ± 0.05 (5)	0.06 ± 0.02 (4)	0.90 ± 0.07 (4)

Sympathetic neurons, ciliary ganglion neurons, and myotubes (2–7 days after plating) were clamped near rest potential (≈60 mV) and AcCho with or without substance P was applied for 10 sec. I_5, inward current level 5 sec after onset of drug(s) application; I_{peak}, peak inward current. The concentration of substance P used was 10 μM except for muscle (40 μM) and with tests of 100 μM AcCho, where 20 μM substance P was applied. All data are mean ± SEM. Number of cells tested is indicated in parentheses. Each condition was tested two times on each cell. SP, substance P.

Examination of the substance P effect on cells clamped at a range of membrane potentials would reveal whether the peptide affects the reversal potential for AcCho action and/or affects other currents activated at more depolarized potentials. Fig. 3 shows the steady-state voltage—current relationship obtained in response to AcCho in the presence and absence of substance P. Peak AcCho current varied linearly with holding potential, with an extrapolated reversal potential of −7 mV. Substance P alone had no direct effect on inward current at any holding potential tested. Furthermore, the peptide did not substantially affect peak AcCho current or the extrapolated AcCho reversal potential. However, comparison of inward current level 5 sec after AcCho application vs. AcCho and substance P reveals a decrease by a factor of ≈13 in the slope conductance in the presence of peptide.

When some neurons were clamped at depolarized potentials (−40 or −30 mV) in addition to the usual effect of substance P on AcCho current duration, peak inward current with AcCho plus substance P was greater than the peak current in response to AcCho alone. Substance P block of an outward current (I_m, measured at depolarized potentials) has been described in sympathetic ganglion neurons of bullfrog (18). A similar phenomenon in the chicken could explain this result.

To determine whether substance P affected the activation of other nicotinic cholinergic receptors, chicken ciliary ganglion neurons [which receive cholinergic and substance P in-

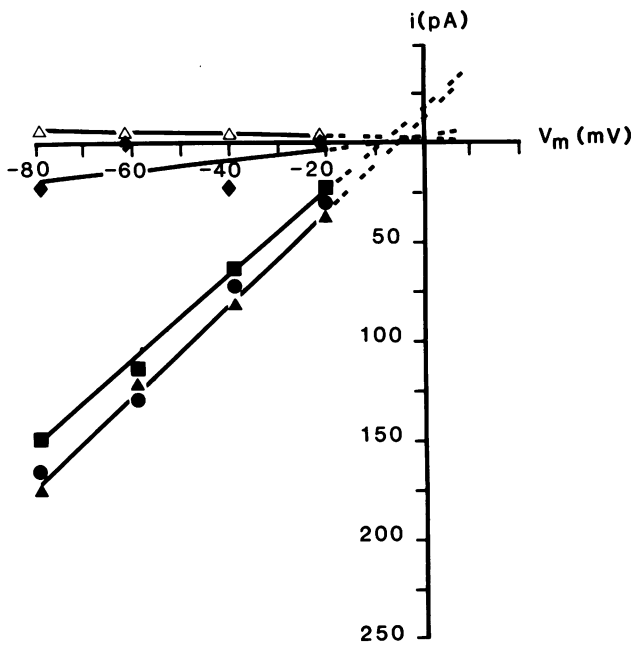

FIG. 3. Current–voltage relationship for substance P inhibition of AcCho-induced currents. Sympathetic neurons were clamped to the indicated holding potentials and the inward current responses to 10-sec application of AcCho with or without substance P were recorded for measurement of peak inward current (I_{peak}) and current level 5 sec after drug application (I_5). (△) I_{peak}, substance P (10 μM); (▲) I_{peak}, AcCho (10 μM); (●) I_{peak}, AcCho (10 μM) and substance P (10 μM); (■) I_5, AcCho; (◆) I_5, AcCho (10 μM) and substance P (10 μM). Note the 13-fold difference in slope conductance with substance P application without significant shift in AcCho-reversal potential. Data presented here are from a neuron that was representative of the five cells tested. Lines drawn are linear regressions of data.

put *in vivo* (7, 8)] and chicken myotubes were also tested. Fig. 4 shows a recording of a typical response of a ciliary ganglion neuron, clamped at rest potential, to 10 μM AcCho in the presence and absence of substance P. Like the sympathetic neurons, the peak current response to AcCho is not substantially affected by concurrent application of the peptide, but the AcCho-induced inward current rapidly decays to baseline if substance P is present. I_5/I_{peak} is typically less by a factor of 14 in the presence of peptide (Table 1). In contrast, substance P has little or no effect on AcCho-induced current decay in chicken skeletal muscle cells (Table 1), suggesting that the peptide modulation may be selective for ganglionic, as opposed to muscle, AcCho receptors.

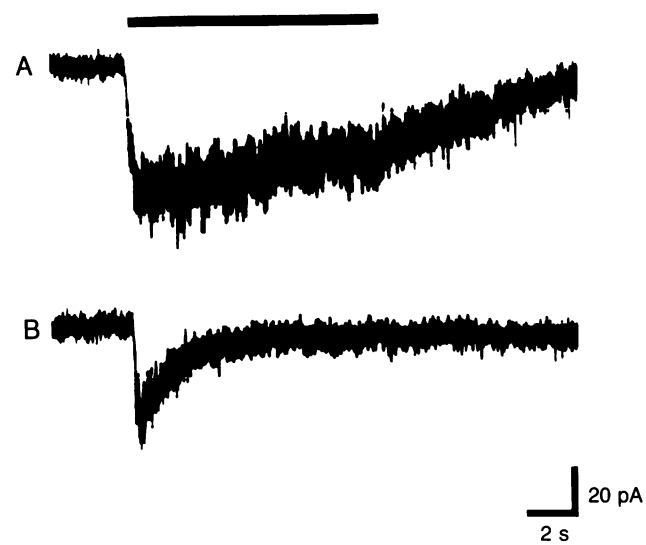

FIG. 4. Substance P increases the rate of decay of AcCho-induced currents in ciliary ganglion neurons. Inward current responses in voltage-clamped ciliary ganglion neuron (V_m = −60 mV). Duration of drug application is indicated by bar. (*A*) 10 μM AcCho; (*B*) 10 μM AcCho and 10 μM substance P.

DISCUSSION

Embryonic chicken sympathetic and ciliary ganglion neurons responded to a prolonged (10-sec) application of AcCho with an initially brisk depolarization (activation of inward current) that decayed with continued exposure to agonist (Fig. 1–3). In contrast to the numerous studies of receptor desensitization at the nerve–muscle synapse (see refs. 25–28 for review), very little is known about desensitization of ganglionic receptors. The data presented here demonstrate that the decay time of the AcCho-induced current in ganglion cells is dependent on agonist concentration (Table 1), similar to agonist-induced receptor desensitization at the nerve muscle synapse (29).

Substance P alone had no effect on resting membrane potential, input impedance, or action-potential duration (Fig. 1 and data not shown). When the peptide was applied concurrently with AcCho to neurons voltage-clamped at rest potential, however, it dramatically enhanced the rate of decay of the AcCho-induced current. This effect of substance P, as well as the decrease in AcCho-induced current "noise" in the presence of substance P (Figs. 2 and 4), is consistent with an enhancement of AcCho-receptor desensitization.

These results are different from a recent report on bullfrog sympathetic ganglia in which, after a 2- to 3-min bath application of substance P, the peptide apparently decreased the peak current response to iontophoretically applied AcCho by ≈40% with no effect on the time course of current decay (14). The discrepancy between the results is difficult to resolve given the different preparations studied. One advantage afforded by studying isolated neurons *in vitro* is the ability to rapidly apply drugs at known concentrations directly to the cell of interest. It is possible that the iontophoretic application of AcCho to ganglion cells *in situ* resulted in local concentrations of AcCho sufficient to induce significant receptor desensitization. Prolonged bath application of substance P might enhance the receptor desensitization, thereby diminishing the peak-current response. Alternatively, the effects of substance P may be quite different in frog and chicken. In experiments reported here, application of substance P just prior to the exposure to AcCho and substance P decreased peak current by ≈20% compared with control. This small decrease in peak current indicates that the peptide may exert some effect on AcCho-induced currents in the absence of prior exposure to cholinergic agonists.

Previous reports in frog and guinea pig sympathetic ganglia have shown a direct depolarizing action of substance P (15–18), probably due to inactivation of an outward current activated at depolarized potentials (I_m, see ref. 18). Although in the chicken substance P had no effect on resting membrane potential or input impedance, the effect of the peptide on membrane currents activated by depolarization needs to be examined more extensively.

The observed effects of substance P on the rate of decay of AcCho-induced currents in chicken autonomic neurons are compatible with several other previous reports. Substance P has been shown to inhibit AcCho-stimulated catecholamine release from mammalian chromaffin cells (3, 4) as well as to decrease the rate of carbachol-stimulated Na^+ influx in PC12 cells (5). In the latter study, inhibition by substance P of carbachol-induced ion flux was consistent with the peptide acting at a regulatory site (i.e., not binding to the

ion channel) and enhancing the rate of agonist-induced receptor desensitization.

In the present study, direct examination of macroscopic currents in voltage-clamped neurons afforded both improved time resolution and the ability to study AcCho-activated currents uncontaminated by voltage-sensitive currents. In contrast to the release and flux studies, therefore, these studies provide a direct measure of the substance P modulation of cholinergic receptor function. To extend these studies and test the hypothesis that substance P potentiates AcCho-receptor desensitization, the phenomenon of agonist-induced desensitization of ganglionic receptors must be studied in more detail. Single-channel current recording might reveal changes in the pattern of channel openings that have been shown to occur with receptor desensitization at the nerve–muscle synapse (30).

Although an increase in receptor desensitization is consistent with the observed results, other possible mechanisms should also be examined. Substance P may decrease the net AcCho-induced current with a slow onset by (*i*) decreasing channel conductance, (*ii*) decreasing mean channel open time, and/or (*iii*) blocking AcCho-receptor channels after they have opened. These possibilities can be readily tested with single-channel current recording. While this work was in progress, substance P modulation of AcCho currents in bovine chromaffin cells was examined with this approach (31).

Data presented here indicate that the substance P modulation of AcCho current decay occurs with chicken sympathetic and ciliary ganglion neurons but not with skeletal muscle cells. A lack of effect of substance P at the chicken nerve–muscle synapse has also recently been reported by Ryall (ref. 32; but cf. ref. 14). Other aspects of the pharmacology of neuronal and muscle AcCho receptors have been noted to be significantly different, including susceptibility to certain antagonists such as α-bungarotoxin. Perhaps modulation of the AcCho receptor by substance P represents another such difference.

Substance P is present in the cholinergic preganglionic neurons that innervate both the paravertebral sympathetics and the ciliary ganglion of the chicken (7–9). This finding, combined with the demonstration that substance P can alter the responses of both of these autonomic neurons to applied AcCho, suggests a possible physiological role for the peptide in modulation of synaptic function. Nicotinic blockade consistent with desensitization at ganglionic synapses has been demonstrated with high-frequency preganglionic firing (33, 34) and Magleby and Pallotta (35) have described synaptic desensitization at the nerve–muscle junction with repeated stimuli to the presynaptic nerve separated by as much as 25 msec. If such levels of presynaptic activation can occur at autonomic ganglia *in vivo*, the physiological role of substance P in the sympathetic and ciliary ganglion may be to decrease postsynaptic responsiveness to AcCho during periods of high presynaptic activation. The plausibility of this mechanism for substance P action can be tested directly at synaptic contacts between pre- and postganglionic neurons *in vitro* and *in vivo*.

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