Peptide Modulation of ACh Receptor Desensitization Controls Neurotransmitter Release From Chicken Sympathetic Neurons

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SUMMARY AND CONCLUSIONS

1. The distribution and release of substance P (SP) in embryonic chicken lumbar sympathetic ganglia was examined with the use of immunohistochemistry and radioimmunoassay, respectively. SP immunoreactivity was detected in nerve fibers surrounding individual sympathetic neurons and was released by ganglionic depolarization.

2. Effects of SP on nicotinic acetylcholine receptor (AChR) function were assayed in embryonic sympathetic neurons in vitro by whole-cell patch clamp. SP (0.1–20 μM) accelerated the rate of decay (desensitization) of ACh-induced currents. The AChR desensitization time course is biphasic and described by the sum of two exponential functions dependent on agonist concentration (time constant of the faster component, τf = 1–2 s, and the slower time constant, τl = 10–25 s). SP selectively decreased τl and the contribution of the slow component to the overall rate of current decay. The effects of SP on desensitization were concentration dependent and reversible. SP slowed recovery from desensitization by 2.5-fold.

3. SP shifted the dose-response curve for ACh-induced desensitization, reducing the concentration of ACh required to produce half-maximal desensitization by approximately twofold.

4. Preapplication of SP was equivalent to SP applied together with ACh in accelerating AChR desensitization. SP did not alter the time course of currents elicited by nondesensitizing concentrations of ACh, carbamylcholine (CARB), or dimethylphenylpiperazinium (DMPP). These data suggest that AChR activation is neither necessary nor sufficient for the peptide to modulate receptor function. A kinetic model of the effects of SP on specific steps in AChR desensitization is presented.

5. SP enhanced the rate of decay of synaptic currents in sympathetic neurons innervated in vitro, decreasing the synaptic current duration by up to 80%.

6. Effects of SP on neurotransmitter release from sympathetic neurons were evaluated by measuring the release of [3H]-norepinephrine (NE). ACh and CARB stimulated NE release in a concentration- and calcium-dependent manner. SP alone had no effect on NE secretion, but the peptide inhibited NE release induced by ACh or CARB by 40–50%.

7. Although agonists specific for either nicotinic or muscarinic receptors stimulated release of NE, SP selectively inhibited the nicotinic component of transmitter secretion. Thus, SP suppressed NE release induced by DMPP by up to 80% but had no effect on muscarinic or depolarization-induced NE secretion.

8. Parallel studies of the modulatory effects of SP on whole-cell currents and NE secretion revealed that SP inhibition of transmitter release from sympathetic neurons is directly proportional to the extent of potentiation of AChR desensitization. We conclude that SP inhibits neurotransmitter secretion from the cells by enhancing nicotinic AChR desensitization.

9. The ganglionic peptide somatostatin (SOM, 2.5–50 μM) also regulated the slow phase of AChR desensitization. The effects of SOM on desensitization were concentration dependent, reversible, and were not additive with those of SP.

INTRODUCTION

The discovery of a variety of small peptides in central and peripheral neurons has stimulated many studies of their possible roles in synaptic transmission. Neuropeptides may behave like classical transmitters, directly altering membrane conductance, or they may play a more modulatory role, altering the presynaptic release of postsynaptic action of other neurotransmitters (for review see Swope et al. 1992). This latter role is particularly interesting because neuropeptides coexist with classical transmitters in many vertebrate neurons (for review see Lundberg and Hokfelt 1983).

The neuropeptide substance P (SP) is widely distributed in the vertebrate CNS (for a review see Pernow 1983) and is present in sensory (New and Mudge 1986), sympathetic (Hokfelt et al. 1977; Matthews and Cuello 1982; New and Mudge 1986), parasympathetic (Bowers et al. 1986; Erichsen et al. 1982) and enteric ganglia (Costa et al. 1981), and in the adrenal medulla (Pfister and Gorne 1983). Both the presence of SP in the autonomic nervous system and the demonstrated effects of applied SP on autonomic neurons suggest a role for SP in the regulation of autonomic transmission. SP can directly depolarize sympathetic neurons (cf. Dun and Minota 1981; Ramirez and Chiapinelli 1987; Tsunoo et al. 1982), apparently by inhibiting potassium conductance (Adams et al. 1983). SP can also modulate the time course of acetylcholine (ACh) responses in sympathetic and parasympathetic (ciliary ganglion) neurons (Bowers et al. 1986; Margiotta and Berg 1986; Role 1984a, b) perhaps by enhancing nicotinic acetylcholine receptor (AChR) desensitization (Boyd and Leeman 1987; Clapham and Neher 1984; Simmons et al. 1990; Stallcup and Patrick 1980). In contrast to the relatively well-documented depolarizing effects of SP, however, the modulatory actions of SP are less well understood, and the physiological significance of the modulation is not at all clear.

In the studies presented here, we demonstrate the presence and release of SP in embryonic chicken sympathetic ganglia where we then characterize the modulatory effects of SP on ACh-induced desensitization in detail. SP enhances the rate of decay of both synaptic currents and currents evoked by cholinergic agonists. The consequences of AChR modulation by SP are evident in a depression of
neurotransmitter release from the sympathetic neurons that is related directly to the extent of SP modulation of AChR desensitization. Because SP is present in and released from embryonic chicken sympathetic ganglia and the peptide inhibits synaptic activation and transmitter release from sympathetic neurons in vitro, AChR modulation by SP may regulate autonomic function in vivo. Some of these results have been presented previously in abstract form (Downing et al. 1987; Role 1984a; Valenta et al. 1987).

METHODS

Cell culture

Neurons were dispersed from embryonic day (ED) 9–12 chicken lumbar sympathetic ganglia and maintained in vitro as previously described (Role 1984b). For secretion experiments, the cell suspension was γ-irradiated (~5,000 rads) before plating to eliminate nonneuronal cells. Cultures were prepared with the use of 0.75–1.0 sympathetic chain, equivalent to ~1–2 × 10^5 cells, per 35-mm culture dish or 16-mm culture well (for electrophysiology and biochemistry, respectively). Sympathetic neurons were innervated by coculture with microexplants of the sympathetic preganglionic nucleus from E8 chick embryos as described by Role (1988) with the modifications described in Gardette et al. 1991. Cultures were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 5% ED 10–11 chick embryo extract (CEE), 10% heat-inactivated horse serum, 2 mM glutamine, 50 U/mL penicillin, 25 mg/mL streptomycin, and 100 ng/mL nerve growth factor (NGF, gift of P. Osborne and G. Johnson, Washington University, St. Louis, MO) and used for experiments 4–8 days after plating.

Electrophysiology

Whole-cell patch-clamp recording used conventional techniques (Hamill et al. 1981). Briefly, cultures were rinsed free of growth medium, incubated in recording medium and placed on the stage of an inverted microscope (Zeiss) at room temperature (22–24°C; except studies of innervated neurons, which were done at 37°C). Patch electrodes prepared from borosilicate glass and filled with internal recording medium (resistance, ~8–12 MΩ) were used to voltage clamp the neurons to −60 mV, unless otherwise noted, using DAGON 8900 or List EPC7 patch-clamp amplifiers. Cholinergic agonists were applied in external recording media for 10 s, unless otherwise noted, by pressure ejection (~10–12 psi) from a 1- to 4-μm-diam pipette placed ~50 μm from the cell soma. The agonist-evoked currents, previously shown to be entirely mediated by nicotinic AChRs (Role 1984b), were monitored on a Nicolet storage oscilloscope and stored on VCR tape with a Neuordata DR4 PCM digitizer.

The kinetics of AChR desensitization were analyzed from the time course of currents elicited by prolonged applications of cholinergic agonist with the use of a nonlinear, least-squares curve-fitting computer program that fitted the decay phase of the currents with the sum of two exponential curves according to the equation
\[ I(t) = I_a \exp(-t/\tau_a) + I_b \exp(-t/\tau_b) \]
where \( I_a \) and \( I_b \) denote the amplitudes of the fast and slow kinetic components at the time of peak current amplitude, and \( \tau_a \) and \( \tau_b \) are the corresponding fast and slow time constants, respectively (algorithms written by the late Dr. S. Schuetze; see Downing and Role 1987). Desensitization was also quantified as the percent decay of the macroscopic current from peak at 5 s, \( I_a \), according to the equation
\[ I_a \% = \frac{I_a(t) - I_a(0)}{I_a(t)} \times 100 \]
where \( I_a \) denotes the peak current amplitude and \( I_a \) denotes current amplitude 5 s after the onset of agonist application, as described in Downing and Role (1987). Data are expressed as means ± SE of determinations made on at least three cells per condition. Control values were normalized where indicated for comparison of data between experiments or for comparison of the early kinetic components of evoked currents uncontaminated by the slow time constant of recovery from desensitization (see RESULTS and Fig. 5). The half-maximal concentrations of ACh or peptide (EC50) were calculated from current decay dose–response curves by linear regression of all values between 20 and 80% of the maximal effect observed (cf. Han et al. 1987).

**SP immunocytochemistry**

The distribution of SP in sympathetic ganglia was examined by the use of standard immunohistochemical methods. Briefly, the lumbosacral region of the spinal cord with attached dorsal root and sympathetic ganglia was dissected from ED 10 or 20 chicken embryos as a single block of tissue and fixed in 4% paraformaldehyde (in 0.1 M phosphate buffer, pH 7.4) for 2–4 h at 4°C. The tissue was then rinsed three times with phosphate buffer and immersed in ice-cold 30% sucrose in 0.1 M phosphate buffer for 24 h. The tissue blocks were frozen in optimal cutting temperature embedding medium (Miles Scientific), and 12–15 μM cryostat sections were collected onto gelatin-coated slides. The sections were incubated overnight at 4°C with rabbit anti-SP antiserum (gift of J. Garcia-Arraras, University of Puerto Rico, Rio Piedras, Puerto Rico) diluted 1:500–1:1000 in phosphate-buffered saline (PBS) containing 0.1% Triton X-100 and 1% normal goat serum. They were then rinsed with PBS containing 1% normal goat serum and incubated for 30–60 min with fluorescein-labeled goat anti-rabbit IgG (TAGO, 1:200; in PBS containing 1% normal goat serum). After rinsing with phosphate buffer, the sections were covered with glycerol (50% in 0.2 M carbonate buffer, pH 9.0) containing 0.04% paraformaldehyde (PPDA) or with Gel/Mount (Biomedia) and viewed with a Zeiss Axioplan microscope equipped with epifluorescence. Photographs were taken on Ilford HP5 black and white film (800 ASA).

**SP release**

The release of SP from lumbar ganglia was monitored by peptide radioimmunoassay (RIA). The L1–L4 ganglia from ED 18 embryos were used for these experiments because of their higher content of SP (Hayashi et al. 1983). Ten sympathetic chains (~30 ganglia) were excised, desheathed, separated into individual ganglia, and preincubated with external recording medium containing 0.1% bovine serum albumin (BSA: RIA grade) and 2 μM bacitracin for 20–200 min. SP release was measured during two consecutive 10-min incubations each in control medium, high K+ medium (56 mM), and control medium. KCl was exchanged for NaCl to maintain osmolarity. After each release period, the incubation media was collected, and 400-μl samples were extracted with 2 M acetic acid/2.5% β-mercaptoethanol and rapidly frozen in dry ice/acetone. The samples were then lyophilized, resuspended in gelatin-containing buffer, centrifuged to remove insoluble material, and assayed by Drs. Takeda and Krause for SP by RIA as described in MacDonald et al. (1989). The limit of detection of the assay is ~2 pg of SP/assay tube, equivalent to ~1% of the average SP content of the samples.

**Norepinephrine (NE) secretion**

Neurotransmitter release from sympathetic neurons was monitored by measuring the release of [3H]norepinephrine ([3H]NE) by modification of the methods of Greene and Rein (1978). Cultures were preincubated with 10 μM pergolide (15 min, 37°C) to inactivate monoamine oxidase, rinsed once with culture media
lacking CEE, and then incubated in 300 μl of media (pH 7.4 in 5% CO₂) containing 1 μCi/ml I-[3H]NE (specific activity, 14.2–19.7 Ci/mM; New England Nuclear), 1 mM ascorbate, and 100 μM EDTA (to inhibit NE oxidation). After 2–3 h, the cultures were rinsed three times and equilibrated in medium alone for an additional 2–3 h. The neurons were then rinsed with Krebs-Ringer saline containing 10% horse serum or 0.5% BSA and transferred to a 37°C H₂O bath. After 15–30 min preincubation, the cells were washed and incubated under the conditions indicated in the text (5 mins, 37°C with gentle agitation). Bacticacin (10 μM) and neostigmine (10 μM) were included in experimental solutions to inhibit SP and ACh hydrolysis, respectively. At the end of the test incubation, the media was collected, the cultures washed with icecold buffer and lysed with 0.1% digitonin (15 min, room temperature). After a final rinse with detergent to collect residual [3H]NE, 200 μl aliquots of the media and cell samples were assayed with the use of a Beckman LSC-9800 scintillation counter. Control experiments indicate that >90% of the radiolabeled material released under these conditions are authentic NE (cf. Greene and Rein 1978; Role et al. 1981).

NE release was quantitated for each culture as a percent of total [3H]NE content [3H] secreted/[3H] remaining in the cells) and is presented as means ± SE of determinations on three to four cultures per condition per experiment. Where indicated, control values were normalized to permit comparison between experiments. Net agonist-stimulated release was calculated by subtracting mean basal release from release values obtained in response to agonist. EC₅₀ values for dose-response curves were calculated by linear regression as described above.

Solutions

External recording medium contained 140 mM NaCl, 6 mM KCl, 0.8 mM MgCl₂, 10 mM CaCl₂, 5 mM glucose, 30 μM phenol red, and 12.5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.4; internal recording medium contained (in mM) 140 KCl, 2 MgCl₂, 11 K₂EGTA, 1 CaCl₂, 5 MgATP, and 10 HEPES; pH 7.2. Krebs-Ringer saline consisted of 137.5 mM NaCl, 4.8 mM KCl, 2.4 mM MgSO₄, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂, 5 mM glucose, 30 μM phenol red, 12.5 mM HEPES; pH 7.4. All solutions containing ACh, pargyline, and bacticacin (Sigma) were prepared the day of the experiment. Tachykinin peptides and somatostatin (Sigma, Peninsula or Bachem) were stored in 0.1 N acetic acid at -20°C and were thawed and diluted just before use. Carbamylcholine (CARB), dimethylphenylpiperazinium (DMPP), muscarine, veratridine, and MgATP were obtained from Sigma. All other chemicals used were reagent grade.

Statistics

All differences between sample means were analyzed by the use of the two-tailed Student’s t test (Zar 1984), except as noted. A probability (P) value of <0.05 was required for significance.

RESULTS

Presence and release of SP in embryonic chicken sympathetic ganglia

SP-like immunoreactivity (SPLI) was observed in the lumbar sympathetic chain in nerve fibers and terminals at both ED 10 and ED 20. At ED 10, a few stained fibers were observed, however, the number of stained fibers increased substantially with ganglionic development. By ED 20, many SP-immunoreactive nerve fibers and terminals were observed in the ganglia that were in close proximity to the cell bodies of the ganglionic neurons (Fig. 1A). No SP-positive cell bodies were detected. SPLI was also seen in the adjacent dorsal root ganglia (DRG) in the somas of a subpopulation of the DRG neurons. Inspection at lower magnification suggested that it is this subpopulation of sensory cells that project SP-positive fibers to the sympathetic ganglia (not shown).

The immunoreactive SP present in the sympathetic ganglia could be released by depolarization. Under control conditions, low levels of SP release were observed (<0.25–2% of the total SP content). However, depolarization of freshly dissected ganglia increased SP release by 3- to 10-fold (Fig. 1B). The presence and release of SP in response to depolarization suggests that SP could play a physiological role in embryonic sympathetic ganglia.

Characterization of agonist-induced desensitization and its regulation by SP

KINETICS OF ACH-INDUCED DESENSITIZATION. Effects of various peptides on AChR desensitization were investi-
MODULATION OF NEURONAL AChR DESENSITIZATION

**Figure 2.** Kinetics of acetylcholine (ACh)-induced desensitization in the presence and absence of substance P (SP). A: sample whole-cell currents evoked by the indicated concentrations of ACh in the presence and absence of SP (20 μM). Decay of the currents is biphasic and is best fit by the sum of 2 exponential functions (shown superimposed on the currents) with relatively fast (τ_f) and slow (τ_s) time constants. SP enhanced the rate of decay of the currents by altering the slow phase of desensitization. The peak amplitude of the currents presented were normalized to facilitate comparison of the time course of the currents. Calibration bar, 2 s. B: time constants of exponential fits to macroscopic currents evoked by ACh vs. ACh concentration. Data are from 6–15 neurons/concentration. Faster rates of current decay observed with increasing concentrations of ACh were accompanied by significant decreases in τ_s (P < 0.02–0.01). C: effect of SP on the time constants of the exponential fits to the current decay. Data were obtained from 5–13 cells/concentration. SP treatment significantly decreased τ_s at ACh concentrations >20 μM (P < 0.05).

Gated by analyzing changes in the time course of currents elicited by prolonged applications of cholinergic agonists. Figure 2A (top left) shows a typical response of a voltage-clamped sympathetic neuron (V_h = −60 mV) exposed to 10 μM ACh. The evoked inward current rises rapidly to an initial peak value and then decays slowly toward baseline in the continued presence of agonist. Higher concentrations of ACh evoked larger currents that decayed at a faster rate (e.g., 50 μM, Fig. 2A, bottom left; Fig. 4), reflecting the agonist concentration dependence of AChR desensitization (for review see Adams 1981).

Examination of the AChR desensitization time course revealed that it is biphasic and well fit by the sum of two exponential functions with relatively fast (τ_f) and slow (τ_s) time constants (Fig. 2A, left). As shown in Fig. 2B, the faster rates of decay observed at higher concentrations of ACh were accompanied by a significant decrease in the slow time constant (from 25 to 10 s, P < 0.01) and a diminished contribution of the slow kinetic component (%slow component, hereafter referred to as %S). For example, %S at 5 μM ACh equals 96.5 ± 3 (mean ± SE, n = 4); and at 100 μM ACh, %S equals 46 ± 4 (n = 4, P < 0.001). Alterations in agonist concentration did not significantly affect the fast time constant (τ_f = 1–2 s, Fig. 2B).

**Effects of SP on AChR Desensitization Kinetics.** SP treatment substantially enhanced the rate of decay of ACh-induced currents in sympathetic neurons, as shown in Fig. 2A (right). Further analysis of the decay time course revealed that SP selectively altered the slow component of AChR desensitization. At ACh concentrations ≥20 μM, SP decreased both τ_s (P < 0.05, Fig. 2C) and the relative contribution of the slow component to the overall rate of current decay (%S with 20 μM ACh = 64 ± 7%, n = 4; %S with 20
μM ACh + SP = 37 ± 3%, n = 4, P < 0.02). SP did not alter T, at any concentration of agonist tested, although it should be noted that the drug application system limited our analysis of events faster than 200 ms in rise time. The effect of SP to enhance the rate of current decay was reversible within 5 min after peptide application (not shown).

CONCENTRATION DEPENDENCE AND PEPTIDE PHARMACOLOGY OF SP MODULATION OF DESENSITIZATION. The effect of SP on ACh-induced desensitization was examined over a range of peptide concentrations (Fig. 3). Modulation by SP was detectable at 100 nM, half maximal at 1 μM, and maximal at 5 μM (P = 0.01). The Hill coefficient (nH) calculated from these data was close to 1.0, suggesting that SP interacts with a single population of independent binding sites to regulate nicotinic receptor function.

To further delineate the binding site mediating effects of SP on neuronal AChRs, a series of structurally related tachykinin peptides was tested for effects on receptor desensitization. Neither physalaemin, eledoisin, nor substance K (neurokinin A) had any effect on ACh-evoked macroscopic current, even at high concentrations (20 μM). Furthermore, [D-Arg1, D-Pro4, D-Trp7,9, Leu11]-substance P, which antagonizes effects of SP in some systems, did not block the effects of SP; instead, this analogue significantly increased the rate of decay of the agonist-induced currents. These data agree with our previous single-channel studies in sympathetic neurons (Simmons et al. 1990) and indicate that the binding site involved in modulating neuronal AChR function has a different tachykinin pharmacology than the neurokinin-1 (NK-1), NK-2, and NK-3 tachykinin receptors described in other systems (for review see Helke et al. 1990).

EFFECT OF SP ON THE CONCENTRATION DEPENDENCE OF ACh-IN- DUCED DESENSITIZATION. To determine whether SP shifts the agonist dependence of AChR desensitization, dose-response curves for the rate of decay of ACh-evoked currents were determined in the presence and absence of SP (Fig. 4). The concentration of ACh required to cause half-maximal AChR desensitization, Kdcsens, was calculated as described in METHODS. Under control conditions, Kdcsens was 15.2 μM ACh. SP treatment shifted the desensitization dose-response curve to the left (Fig. 4B), decreasing Kdcsens to 7.6 μM.

Mechanism of SP modulation of neuronal AChR function

LACK OF VOLTAGE DEPENDENCE OF SP REGULATION OF DESENSITIZATION. SP is positively charged at neutral pH (reviewed in Pernow 1983), so that the peptide might appear to increase the rate of AChR desensitization by direct open channel block of the AChRs in a voltage-dependent manner. In test this, the rate of AChR desensitization was measured over a range of holding potentials in the presence and absence of the peptide. These experiments indicate that the rate of decay of ACh-evoked currents is voltage independent (at −40 mV, I50 = 72.7 ± 2.7, n = 7; and at −110 mV, I50 = 76.1 ± 6.4%, n = 14) and that the effect of SP on AChR desensitization is unaffected by large changes in
TABLE 1.  SP potentiates but does not induce AChR desensitization

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Control</th>
<th>+SP</th>
<th>%Current Decay (I_5)</th>
</tr>
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<tbody>
<tr>
<td>ACh (1 μM)</td>
<td>-0.3 ± 1.6 (20)</td>
<td>3.5 ± 1.9 (29)</td>
<td></td>
</tr>
<tr>
<td>ACh (10 μM)</td>
<td>25.5 ± 2.7 (19)</td>
<td>51.9 ± 4.7* (27)</td>
<td></td>
</tr>
<tr>
<td>CARB (20 μM)</td>
<td>2.2 ± 1.4 (9)</td>
<td>4.8 ± 1.3 (9)</td>
<td></td>
</tr>
<tr>
<td>CARB (500 μM)</td>
<td>36.0 ± 3.0 (15)</td>
<td>81.0 ± 2.0* (9)</td>
<td></td>
</tr>
<tr>
<td>DMPP (1 μM)</td>
<td>17.0 ± 1.0 (8)</td>
<td>21.0 ± 2.0 (8)</td>
<td></td>
</tr>
<tr>
<td>DMPP (10 μM)</td>
<td>39.0 ± 2.0 (5)</td>
<td>86.0 ± 2.0* (4)</td>
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</table>

Data are means ± SE of determinations made on the number of cells indicated in parentheses. Effects of SP (20 μM) on the rate of decay of currents evoked by desensitizing (high) and non desensitizing (low) concentrations of the indicated cholinergic agonists. SP, substance P; AChR, acetylcholine receptor; ACh, acetylcholine; CARB, carbamylcholine; DMPP, dimethylphenylpiperazinium. *Significantly different from control (agonist alone) at P < 0.001.

transmembrane potential (i.e., at -40 mV, I_5 = 77.8 ± 4.7%, n = 11; and at -110 mV, I_5 = 81.0 ± 7.6%, n = 6).

LACK OF EFFECT OF SP ON CURRENTS EVOKED BY NONDESENSITIZING CONCENTRATIONS OF AGONIST. To further test whether SP might directly block AChR channels rather than accelerating AChR desensitization, the effect of the peptide on currents elicited by desensitizing versus nondesensitizing concentrations of various cholinergic agonists were compared (Fig. 4 and Table 1). SP significantly enhanced the rate of decay of currents evoked by high, desensitizing concentrations of ACh, CARB, or DMPP (P < 0.05~0.001). However, the peptide had no effect on either the peak or the decay of currents evoked by low, nondesensitizing concentrations of each of these agonists. These results suggest that AChR activation per se is not sufficient for SP to alter AChR function, and that SP potentiates, but does not induce, the transition from the open to the desensitized state(s) (see APPENDIX).

PERSISTENCE OF SP EFFECTS IN THE ABSENCE OF PEPTIDE. Although the results presented above indicate that SP can enhance AChR desensitization within seconds of AChR activation, additional experiments revealed that AChR channel opening in the presence of the peptide is not required. After determination of the control rate of AChR desensitization, neurons were exposed to SP in the absence of agonist for 10 s. The peptide was removed by local superfusion, and, after 1–3 min, AChR desensitization evoked by ACh alone was measured again. Under these conditions, SP significantly increased the rate of desensitization elicited by a second application of agonist (ACh alone, I_5 = 42.3 ± 6.3%, n = 8; ACh alone 1–3 min after SP, I_5 = 63.9 ± 4.6%, n = 6, P < 0.05). No change in the rate of desensitization was observed if buffer was applied instead of SP. Thus SP modulation of AChR function does not require that the peptide interact with open AChR channels as predicted if the peptide were acting as an open channel blocker. Furthermore, the persistence of SP effects over several minutes in the absence of peptide are in accord with previous results suggesting the involvement of second messenger mechanism(s) in SP action (e.g., see Downing et al. 1987; Harish 1989; Simmons et al. 1990).

EFFECT OF SP ON RECOVERY FROM DESENSITIZATION. Although the rate of decay of ACh-evoked currents remained constant with repeated applications of agonist, the peak amplitude of the currents declined substantially with
repeated agonist exposure, especially at high concentrations of agonist (see Fig. 5A). This steady decline in the magnitude of currents evoked by agonist alone prevented a reliable analysis of SP effects on peak current. Because the peak of the currents did not decline in neurons maintained in the whole-cell configuration for 25 min between agonist applications (not shown), the decrease was apparently determined by the extent of agonist exposure, rather than a time-dependent "washout" of the whole-cell response. The decline in peak current with repeat agonist applications reflects a slow recovery from desensitization and is discussed in more detail in the APPENDIX.

To examine the kinetics of the more rapid phases of recovery from AChR desensitization, ACh was applied to sympathetic neurons for 10 s to obtain an initial peak current value, and then, after various intervals of time, a second response was elicited and compared with the initial response. When high concentrations of agonist were used (e.g., 100 μM ACh), no recovery of peak current amplitude was observed in response to several applications of agonist over many minutes (Fig. 5A), suggesting either an irreversible loss of a portion of the activatable receptors on the cell surface (cf. Boyd 1987) or the existence of a long-lived desensitized state (see APPENDIX). SP treatment further enhanced the loss of agonist sensitivity at all time points assessed, as shown (Fig. 5A, P < 0.05-0.02).

When the lower agonist concentrations (e.g., 20 μM ACh) were used in this paradigm and the duration of the second ACh application was decreased to 2 s, recovery from desensitization was observed. The amplitude of the second response to ACh alone returned to that of the first with a half time of ~50 s (Fig. 5B). SP slowed this recovery; the peak current evoked by ACh plus SP recovered with a half time of ~150 s.

Physiological impact of SP modulation of AChRs in sympathetic neurons

SP DECREASES THE DURATION OF SYNAPTIC CURRENTS. To determine whether SP alters synaptic activation of sympathetic neurons, effects of the peptide were evaluated in cells that were innervated in vitro by explants containing the sympathetic preganglionic nucleus. Under these conditions, spontaneous synaptic currents are recorded from the sympathetic neurons that are mediated entirely by nicotinic cholinergic synapses (Gardette et al. 1991; Role 1988). Effects of SP on the amplitude and decay of the synaptic currents were evaluated (50-100 synaptic currents/cell ± SP). SP enhanced the rate of decay of the synaptic currents, where the average duration of 50-100 control synaptic currents, measured from the t1/2, of the rise of the synaptic current to t1/2 of the decay, was compared with the same parameter for 50-100 synaptic currents evoked after treatment with SP (decreases in synaptic current half duration of 80, 75, 41, 23, 22, 18, 17, 7, and 0%; Fig. 6). In some experiments SP also increased the frequency of synaptic currents, although no effect on synaptic current amplitude was noted (data not shown).

**EFFECT OF SP ON NEUROTRANSMITTER RELEASE.** To determine whether SP modulates transmitter release from sympathetic neurons, we studied the release of [3H]norepinephrine ([3H]NE) in cultured sympathetic neurons by a modification of the protocol of Greene and Rein (1978). The assay provided a reliable measure of both receptor-mediated and depolarization-induced transmitter secretion from these neurons in vitro.

Cholinergic agonist-stimulated efflux of [3H]NE: dose and calcium dependence. In the absence of agonist, only low levels of spontaneous or basal release of [3H]NE were observed (Fig. 7A), which varied from 1 to 8% of the total radioactivity associated with the cells and averaged 4.2 ± 0.3% (n = 90). ACh (100 μM) and CARB (1 mM) evoked 2- to >9-fold increases in NE release from the neurons (Fig. 7A). The acetylcholinesterase inhibitor neostigmine (10 μM), required when ACh was used as agonist, had no effect on basal release of [3H]NE (not shown).

Stimulation of NE release by CARB was detected at con-
MODULATION OF NEURONAL ACHR DESENSITIZATION

Fig. 7. Cholinergic agonist stimulation of neurotransmitter release from sympathetic neurons: dose and calcium dependence. A: norepinephrine (NE) secretion stimulated by the indicated cholinergic agonists [Basal, saline alone; 100 μM acetylcholine (ACh); 1 mM carbamylcholine (CARB)] in control saline (●) and in saline containing 0 added Ca²⁺/10 × Mg²⁺ (▲). Data were obtained in 3 experiments, and the total number of cultures tested is indicated in parentheses. NE release stimulated by ACh and CARB was completely blocked in the absence of extracellular calcium. B: secretion of [³H]norepinephrine ([³H]NE) from sympathetic neurons as a function of CARB concentration. Data were from determinations made in 3 experiments on 3–12 cultures per concentration and normalized to secretion at 1 mM. The concentration of CARB inducing half-maximal secretion, obtained by linear regression as described in METHODS, was 700 PM (r = 1.00).

Centrations as low as 5 μM, half maximal at 700 μM, and approximately maximal at 5 mM (Fig. 7B). Omission of calcium and addition of a 10-fold higher concentration of magnesium reduced the release of NE induced by CARB (1 mM) and ACh (100 μM) to the basal level (Fig. 7A). As shown, saline containing 0 calcium/10 × Mg²⁺ had no effect on basal NE secretion.

SP INHIBITS NEUROTRANSMITTER RELEASE FROM SYMPATHETIC NEURONS. To test whether SP regulates neurotransmitter release from sympathetic neurons, NE secretion was measured in response to ACh and CARB alone and in combination with SP (Fig. 8A). SP inhibited ACh or CARB-stimulated [³H]NE secretion from the neurons by ~50%, whereas SP by itself had no effect on the [³H]NE release (Fig. 8A). The inhibitory effect of SP varied from ~32 to ~66% and averaged ~47% (P < 0.001). Acetic acid (0.001 N), included in SP-containing solutions to enhance peptide solubility, did not affect NE secretion in the absence of peptide: with 100 μM ACh alone, NE secretion equals 7.1 ± 0.2% (n = 8); with ACh + 0.001 N acetic acid, NE secretion equals 7.6 ± 0.4% (n = 8). Although SP can depolarize chicken sympathetic neurons at later stages of development (Ramírez and Chiapinelli 1987), the peptide did not increase NE release from these embryonic neurons (Fig. 8A).

SP HAS NO EFFECT ON TRANSMITTER SECRETION STIMULATED BY NONCHOLINERGIC DEPOLARIZATION. Because NE release from the neurons is dependent on the influx of calcium and SP has been shown to directly inhibit voltage-dependent

Fig. 8. SP inhibits neurotransmitter secretion from sympathetic neurons. A: [³H]NE secretion measured in a representative experiment in response to buffer alone (Basal). SP (20 μM), ACh (100 μM), or ACh in combination with SP. SP inhibited ACh-induced NE release by ~50% (P < 0.001). B: histogram summarizing effects of SP on net agonist-induced NE release evoked by ACh (100 μM) and CARB (0.2–1 mM). Data were obtained in 8 experiments, and the total number of cultures tested for each condition is indicated in parentheses. Solid bars, control release; hatched bars, +SP. SP inhibited ACh and CARB-induced NE secretion by an average of ~40% (P < 0.001). Basal release in these experiments, which was subtracted to obtain net agonist-evoked secretion, averaged 2.6 ± 0.3% (n = 77). For abbreviations, see legends of Figs. 6 and 7.
calcium channels (Bley and Tsien 1990), we tested whether SP inhibition of ACh-evoked NE release was due to AChR modulation and/or calcium channel inhibition. Both the sodium channel agonist veratridine (10–25 μM) and elevated concentrations of K⁺ (30–50 mM) stimulated NE release from sympathetic neurons in a concentration- and calcium-dependent manner (Fig. 9A). The stimulation of NE release by increased [K⁺] (30–40 mM) was blocked by incubation in 0 Ca²⁺/10 mM Mg²⁺ containing medium, whereas veratridine-induced NE secretion was only partially blocked by these conditions (Fig. 9A and data not shown). Neither 0.1% EtOH (the vehicle for veratridine), nor 0 Ca²⁺/10 × Mg²⁺ buffer had any effect on basal NE secretion (Fig. 9A and data not shown).

In the same experiments where SP substantially inhibited ACh-evoked NE secretion, the peptide had no effect on NE secretion when high K⁺ (30 mM) or veratridine were used to depolarize the neurons (Fig. 9B). Thus the peptide does not modulate transmitter release in sympathetic neurons by altering the activity of voltage-gated Na⁺ or Ca²⁺ channels or by inhibiting the exocytotic process per se.

**Inhibitory Effect of SP is Specific for Release Elicited by Activation of Nicotinic AChRs.** In vivo, the ACh released from preganglionic input depolarizes sympathetic neurons via both nicotinic and muscarinic cholinergic receptors (Ramirez and Chiapinelli 1987). To examine whether activation of both receptor types might contribute to ACh-evoked catecholamine secretion, we evaluated the effects of receptor subtype-specific agonists on NE release (Fig. 10).

Both DMPP (5 μM; a selective nicotinic agonist) and muscarine (1 mM) stimulated the release of [³H]NE. Secretion in response to DMPP was approximately half maximal at 5 μM, and maximal at 50 μM (not shown). Lower levels of release evoked by higher concentrations of DMPP suggest that pronounced AChR desensitization occurs under these conditions. Muscarine stimulation of NE release also appeared to be dose dependent over the range of agonist concentrations tested (0.1–1 mM). The finding that both nicotinic and muscarinic receptors are coupled to the secretory pathway in the neurons suggests that activation of both receptor subtypes is likely to contribute to NE release evoked by ACh.

SP inhibited NE release evoked by DMPP (5–10 μM) by ~75% (P < 0.001, Fig. 10B). In contrast, SP had no effect on NE secretion elicited by muscarine (0.1–1 mM, Fig. 10B and not shown). These results indicate that SP inhibits NE secretion by selective modulation of nicotinic AChRs.

**SP Modulation of NE Secretion Correlates with the Degree of AChR Desensitization.** As presented above, the effect of SP to increase the rate of decay of ACh-induced currents is dependent on the extent of agonist-induced desensitization (Fig. 4 and Table 1). If SP inhibits transmitter release by modulating nicotinic receptor desensitization, a similar relationship between SP inhibition of release and agonist-induced desensitization would be predicted. To examine this possibility, the effect of SP on whole-cell currents and NE secretion was examined in sibling cultures with the use of high (desensitizing) and low (relatively nondesensitizing) concentrations of DMPP (Fig. 11). SP significantly enhanced the rate of decay of macroscopic currents (Fig. 11A) and inhibited NE release (Fig. 11B) evoked by a desensitizing concentration of DMPP (e.g., 10 μM, P < 0.05). In contrast, at low concentrations of DMPP that elicited little AChR desensitization (e.g., 1 μM), SP effected neither the DMPP-evoked currents nor the DMPP-evoked NE secretion. These data indicate that the SP-induced inhibition of neurotransmitter release is due to the effect of the peptide on AChR desensitization.

**Modulation of AChR Function by Somatostatin**

The neuropeptide somatostatin (SOM) is also present in embryonic chicken sympathetic ganglia (Havashi et al. 1983; New and Mudge 1986) and has been localized to the cell bodies and nerve terminals of small intensely fluorescent (SIF) cells (J. Garcia-Arraras, personal communication), a class of interneurons present in a chick sympathetic
ganglia. The modulation of NE release by SOM has been compared with similar effects of SP in chromaffin cells (Boksa et al. 1982; Mizobe et al. 1979; Role et al. 1981). Because the effects of these peptides on transmitter release appears to be similar, we tested whether SOM also modulates AChR desensitization in sympathetic neurons.

MODULATORY EFFECTS OF SOM ON ACh-INDUCED DESSENSITIZATION. SOM (20 μM), like SP, enhanced the rate of decay of ACh-evoked currents in sympathetic neurons (Fig. 12A), apparently accelerating AChR desensitization. The effects of these peptides on AChR desensitization are not common to all peptides expressed in the ganglia, however, because micromolar concentrations of both vasoactive intestinal peptide (VIP) and [methionine]-enkephalin, had no effect on the rate of agonist-induced current decay (not shown).

Examination of the effects of SOM on the kinetics of current decay revealed significant decreases in τs (from 25.4 ± 3.2 s, n = 5, to 4.9 ± 3.2 s, n = 5, P < 0.01), decreases in the contribution of the slow component to the overall rate of current decay (from 71.5 ± 4.5%, n = 5, to 23.4 ± 1.5; n = 5, P < 0.01; see Fig. 12A) with no effect on τf. The effect of SOM was also evaluated by the use of desensitizing and nondesensitizing concentrations of agonist. SOM had no effect on ACh responses evoked by nondesensitizing concentrations of agonist. SOM had no effect on ACh responses evoked by nondesensitizing concentrations of agonist. SOM had no effect on ACh responses evoked by nondesensitizing concentrations of agonist.

FIG. 10. Selective effect of SP on NE release mediated by nicotinic AChRs. A: NE release measured in a representative experiment in response to the indicated cholinergic ligands. Both the nicotinic agonist dimethylphenylpiperazinium (DMPP, 3 μM) and the muscarinic agonist muscarine (MusC, 1 mM) stimulated significant NE release from the neurons (P < 0.001). [ACH] = 100 μM, [CARB] = 1 mM. B: summary of effects of SP (70 μM) on NE secretion stimulated by DMPP (5 μM) and muscarine (1 mM). Data were obtained in 8 experiments on a total of 112 cultures and were normalized to NE secretion in response to agonist alone. Control, solid bars; +SP, hatched bars. SP significantly inhibited DMPP-induced NE secretion (P < 0.001) but had no effect on NE secretion induced by muscarine. Basal release averaged 2.3 ± 1.4% (n = 32, subtracted to obtain agonist-dependent secretion). For abbreviations, see legends of Figs. 3 and 7.

FIG. 11. Inhibitory effects of SP on neurotransmitter release correlate with the degree of agonist-induced desensitization. A: sample DMPP-induced currents obtained in response to the indicated concentrations of agonist in the presence and absence of SP (20 μM). Calibration bars, 1.2 pA, 2 s. B: DMPP-induced NE secretion measured in sibling cultures to those recorded from in A. Control, solid bars; +SP, hatched bars. SP significantly enhanced nicotinic AChR desensitization and inhibited NE induced by high, desensitizing concentrations of DMPP (10 μM, P < 0.05). In contrast, SP had no effect on current responses or NE release induced by concentrations of DMPP that elicited minimal agonist-induced desensitization (1 μM). For abbreviations, see legends of Figs. 3, 7, and 10.
concentrations of agonist. For example, with 1 μM ACh, \( I_{\alpha_5} = 1.0 \pm 1.7\% \) (n = 25), and with 1 μM ACh + SOM, \( I_{\alpha_5} = 1.1 \pm 1.0\% \) (n = 18, P > 0.25, 1-tailed t test); in contrast, with 15 μM ACh, \( I_{\alpha_5} = 63.0 \pm 7.1\% \), (n = 9); and with 15 μM ACh + SOM, \( I_{\alpha_5} = 81.6 \pm 5.6\% \) (n = 6, P < 0.05). These data indicate that SOM, like SP, enhances the rate of decay of ACh-induced currents by selectively modulating the slow phase of AChR desensitization.

**Dose-response characteristics of SOM modulation of AChR function.** Modulatory effects of SOM were detected at a concentration of 2.5 μM and were maximal at 50 μM (P < 0.005; Fig. 12). The concentration of peptide eliciting half-maximal effects was calculated as described in Methods. The apparent EC₅₀ obtained, 4.7 μM, is within the wide range of EC₅₀(2–20 μM) reported for SOM inhibition of catecholamine release from bovine and guinea pig chromaffin cells and from the neoplastic rat chromaffin cell line PC12 (Boksa et al. 1982; Mizobe et al. 1979; Role et al. 1981).

**Lack of additivity between the effects of SOM and SP.** Because both SOM and SP are present in the ganglia, the peptides could interact in vivo in their modulation of nicotinic receptor function. To test this idea, high concentrations of SP and somatostatin (20 μM) were tested alone and in combination for effects on AChR desensitization. The presence of one peptide completely prevented any additional effect of the other (% decay = 23.6 ± 1.7 with ACh alone, 48.7 ± 3.7 with ACh + SP, 45.1 ± 4.9 with ACh + SOM, and 42.9 ± 4.1 with ACh + SP + SOM; n = 6–8/condition). The lack of additivity between the peptides suggests that they may share a common step in their mechanism to enhance AChR desensitization.

**Discussion**

**Properties of AChR desensitization in sympathetic neurons**

Desensitization is a property of many neurotransmitter receptors (for review see Huganir and Greengard 1990) and appears to be an important aspect of neurotransmitter-receptor interaction. In the studies presented here the kinetics of neuronal AChR desensitization were investigated by measuring alterations in the rate of decay of macroscopic currents evoked by cholinergic agonists in voltage-clamped sympathetic neurons. These analyses revealed two phases of desensitization: an initial rapid phase that was not detectably affected by agonist concentration, and a slower phase whose rate increased as a function of agonist concentration. Studies of AChR desensitization at the neuromuscular junction have also revealed two or more kinetic components with temporal characteristics similar to those described here (Chestnut 1983; Feltz and Trautman 1982).

**Initial studies of receptor desensitization carried out in the chromaffin cell line PC12** (Boyd 1987) and in autonomic neurons (Downing and Role 1987; Margiotta et al. 1987; Role 1984a,b) also suggest that neuronal AChRs desensitize in a biphasic manner.

Desensitization rates of neuronal nAChRs appeared to be relatively insensitive to alterations in transmembrane voltage. These results are in marked contrast to those obtained in skeletal muscle, where the rate of AChR desensitization increases approximately threefold at hyperpolarized potentials (cf. Magazanik and Vyskocil 1970). This difference may reflect the divergence in primary structure of neuronal versus muscle-type AChRs [see Heinemann et al. (1990) for a review]. Alternatively, our examination of desensitization at the level of the macroscopic ACh currents may mask differences in the voltage dependence of desensitization of the distinct AChR channel subtypes expressed by these neurons (Moss et al. 1989; Moss and Role 1993).

**Peptide modulation of AChR desensitization**

Both SOM and SP are present in chick sympathetic ganglia and substantially increase the decay of ACh-evoked currents in sympathetic neurons. This modulatory effect appears to be due to a selective enhancement of the rate of the slow phase of AChR desensitization. Peptide modulation of desensitization was concentration dependent, reversible, and occurred within seconds of peptide application. In addition, SP treatment decreased the concentration of ACh.
required for half-maximal desensitization by approximately twofold, suggesting that the peptide may stabilize AChRs in a higher affinity, desensitized conformation, and/or facilitate the transition to this state (see APPENDIX).

The data presented suggest that the modulatory effects of SP on AChR desensitization in sympathetic neurons are mediated by a site with micromolar affinity and high structural specificity for SP. These data are in agreement with pharmacological studies of SP effects on chromaffin cells (Boyd and Leeman 1987; Mizobe et al. 1979; Simasko et al. 1985) and indicate that established tachykinin receptor subtypes are not involved in the modulatory actions of the peptide. Micromolar concentrations of SP have also been reported to produce a slow, long-lasting depolarization of chicken sympathetic neurons later in embryonic development (Ramirez and Chiapinelli 1987). Because this effect is mimicked by the tachykinins eledoisin and physalaem in and is blocked by the putative SP antagonist tested in our experiments, the direct and modulatory actions of SP on sympathetic neurons must be mediated by distinct peptide binding sites.

Four lines of evidence suggest that it is unlikely that the peptide alters receptor function via a nonspecific mechanism such as a direct block of the gated AChR channel. 1) Effects of SP (which is positively charged) on AChR desensitization were comparable over a 90-mV transmembrane potential range, thus SP does not block AChR channels in a voltage-dependent manner. 2) Pretreatment with SP before AChR activation enhanced the rate of AChR desensitization with subsequent agonist exposure, whereas an open channel block mechanism would require AChR channel gating in the presence of peptide. Thus AChR channel gating is not necessary for SP action. 3) SP had no effect on macroscopic currents evoked by non-desensitizing concentrations of agonist, thus channel gating is not sufficient for SP action. 4) SP modulates AChR channels in cell-attached patches when the peptide is applied to the extra-patch membrane, i.e., under conditions where the AChR channels are not exposed to the peptide at all (Simmons et al. 1990). Taken together, these experiments demonstrate that SP modulates AChR desensitization through an indirect mechanism, rather than directly blocking ion flux through the receptors. This conclusion is consistent with previous work in PC12 (Boyd and Leeman 1987; Stalleup and Patrick 1980) and chromaffin cells (Clapham and Neher 1984a,b) and autonomic neurons (Margiotta and Berg 1986; Role 1984). In addition, we find that SP slowed recovery from desensitization in sympathetic neurons, an effect of the peptide that has been rather controversial in previous studies of chromaffin cells (e.g., see Stalleup and Patrick 1980; Boyd and Leeman 1987; Clapham and Neher 1984; Lyford et al. 1990).

The modulatory effects of SP on both the onset and recovery from AChR desensitization can be explained by multiple effects of the peptide on the transitions between the activated and desensitized state(s). A kinetic scheme for neuronal AChR desensitization is presented in the APPENDIX. This model accounts for the observed effects of SP on AChR function in sympathetic neurons, indicating that SP potentiates the transition of AChRs from the activated state to two distinct desensitized states as well as inhibiting the recovery from the desensitized to the activatable state(s).

Although our experiments indicate that SP does not modulate AChRs via classical peptide receptors, the involvement of a second messenger in accelerating AChR desensitization is suggested by several findings. First, studies presented here demonstrate that SP modulation of AChR desensitization persists for several minutes in the absence of peptide. Furthermore, our previous studies (Simmons et al. 1990) demonstrate modulation of AChR channel opening frequency and open time in cell-attached patches with SP applied only to the remainder of the cell. A number of lines of evidence implicate phosphoinositide/protein kinase C (PKC) as the likely second-messenger pathway mediating SP modulation of AChR desensitization. First, activators of C kinase precisely mimic the effects of SP on AChR function, enhancing the rate of AChR desensitization, selectively decreasing gating, and the contribution of the slow kinetic component to the overall rate of current decay (Downing and Role 1987). Second, both phosphoinositide turnover and PKC activation is detected within seconds of SP treatment of the neurons (Harish 1989). Most compelling is the observation that SP modulation of AChRs is completely blocked by C kinase inhibitors (Simmons et al. 1990). It is not yet clear how SP stimulates PKC in the neurons, although recent studies of mast cells demonstrate a variety of cationic amphophilic peptides, including SP that directly activate the G proteins G_i and G_s in a receptor-independent manner, stimulating phosphoinositide hydrolysis and, presumably, PKC activation (for review see Mousli et al. 1990). SP may activate PKC in sympathetic neurons by a similar mechanism.

If SP acts via a second-messenger mechanism, why does the modulation require AChR activation by desensitizing concentrations of agonist? Studies of muscle and Torpedo nicotinic AChRs indicate that these receptors can be phosphorylated in the absence of agonist by various second-messenger–activated protein kinases, and that increased phosphorylation accelerates the rate of muscle AChR desensitization (for reviews see Huganir and Miles 1989; Swope et al. 1992). The amino acid sequence of several of the AChR subunits expressed in chicken sympathetic neurons (Isterud et al. 1991) contains consensus sites for C kinase–mediated phosphorylation (Huganir and Greenard 1990). Perhaps, agonist-induced desensitization of neuronal AChRs is necessary to convert the receptors to a conformation in which the putative phosphorylation site is accessible for kinase-mediated phosphorylation. In this scheme, phosphorylation of the site(s) by SP activation of PKC would stabilize the desensitized conformation of the receptors thus slowing their recovery from the desensitized state(s).

Potential physiological role for SP in the regulation of sympathetic ganglion function

We have found that immunoreactive SP is both present in and released from embryonic sympathetic ganglia by depolarization. The peptide is detected in the ganglia in nerve terminals that probably arise from axon collaterals of DRG neurons. SP-positive nerve fibers in sympathetic ganglia of other species (cf. Matthews and Cuello 1982) also appear to be sensory in origin. Our finding that ganglionic SP is released in response to depolarization is consistent with re-
results obtained by Ramirez and Chiapinelli (1987), who found that high-frequency presynaptic stimulation evokes slow synaptic responses in chicken sympathetic neurons that are mimicked by exogenous SP and are blocked by a SP antagonist.

Modulation of nicotinic AChRs by SP alters synaptic function in sympathetic neurons innervated in vitro. SP enhanced the decay and decreased the duration of spontaneous cholinergic synaptic currents. Bowers et al. (1986) obtained similar results in their studies of bullfrog parasympathetic ganglia. In their experiments the ganglionic neurons were activated synaptically by the use of high-frequency presynaptic nerve stimulation. Under these conditions, exogenously applied SP enhanced the rate of repolarization of the resulting excitatory postsynaptic potentials (EPSPs).

The SP-induced decrease in synaptic current duration in sympathetic neurons described here is likely due to the distinct effects of SP on mean channel open time (Simmons et al. 1990). Shortening of the synaptic currents would be expected to decrease summation of EPSPs in the sympathetic neurons, thereby decreasing the net suprathreshold activation of the neurons and hence the net transmission through the ganglia.

The results presented here also suggest that SP modulation of AChR function depresses neurotransmitter release. SP substantially inhibited ACh-induced [3H]NE release from embryonic chicken sympathetic neurons in vitro. Although these results are consistent with previous findings in chromaffin cells (Ivett et al. 1979; Mizobe et al. 1979; Role et al. 1981), our studies provide the first demonstration that SP regulates neurotransmitter release from neurons.

Embryonic chicken sympathetic neurons secrete NE in response to both nicotinic and muscarinic receptor agonists. Although SP significantly inhibited NE release induced by ACh, CARB, and the nicotinic agonist DMPP, the peptide had no effects on secretion evoked by the muscarinic agonists, indicating that SP modulation is selective for nicotinic receptors. These results are also in accord with previous studies in adrenal medullary chromaffin cells (Role et al. 1981; Zhou et al. 1991). SP inhibited catecholamine release evoked by mixed cholinergic agonists such as ACh and CARB by ~40–50%. Because SP did not inhibit the action of muscarinic agonists, it is likely that the fraction of ACh and CARB-induced NE secretion that is resistant to SP is mediated by muscarinic receptors.

The binding of ACh to its receptors in sympathetic neurons is clearly just the first step in a series of events leading to the release of NE. We tested some of the possible mechanisms by which SP might inhibit NE secretion from sympathetic neurons. SP did not alter NE secretion elicited by depolarization with high K+ or veratridine, suggesting that the peptide does not regulate transmitter release by modulating the activity of the Na+ or Ca2+ channels activated by these secretagogues. These results are interesting in light of a report that SP substantially inhibits N-type calcium currents in frog sympathetic neurons (Bley and Tsien 1990). Apparently, depolarization-induced neurotransmitter release from chicken sympathetic neurons does not require significant calcium entry through N-type channels.

It has been proposed that desensitization of nicotinic cholinergic receptors by SP might account for the inhibitory effect of the peptide on catecholamine secretion in chromaffin cells (cf. Role et al. 1981). We have tested this hypothesis directly in neurons by comparing the effect of SP on macroscopic ACh-evoked currents and transmitter release elicited by desensitizing and nondesensitizing concentrations of the nicotinic agonist DMPP. SP substantially inhibited NE secretion from sympathetic neurons when the peptide significantly increased the rate of agonist-induced desensitization but had no significant effect when the peptide did not enhance desensitization. These results suggest that activating nicotinic AChRs in the presence of SP is not sufficient to modulate transmitter release. Rather, modulatory effects of SP on neuronal transmitter secretion result directly from the effect of the peptide to potentiate AChR desensitization.

Conclusions

SP, perhaps released during periods of prolonged ganglionic depolarization (e.g., high-frequency transmission), may potentiate desensitization of nicotinic AChRs in sympathetic neurons, thereby inhibiting subsequent nicotinic transmission and neurotransmitter release from the cell. In both the direct depolarizing (Ramirez and Chiapinelli 1987) and modulatory effects of SP are operative in vivo. SP might act to augment nicotinic responses at low levels of preganglionic activity, and inhibit nicotinic transmission at high frequencies of presynaptic activation, when AChR desensitization is likely to occur.

Appendix

Kinetic scheme for AChR desensitization

The following reaction scheme for desensitization of neuronal AChRs was used to consider the effects of SP on AChR function in more detail.

\[
\begin{align*}
R &\overset{k_1}{\longrightarrow} A_1 R^* \\
D_1 &\overset{k_2}{\longrightarrow} A_2 D_1 \\
A &\overset{k_3}{\longrightarrow} A_2 D_2 \\
A_2 R^* &\overset{k_4}{\longrightarrow} A_2 R^* \\
D_2 &\overset{k_5}{\longrightarrow} A_2 D_2 \\
(A1)
\end{align*}
\]

In this scheme the unliganded receptor (R) combines with two molecules of agonist (A) and undergoes a transition from the closed-ligated (A1,R) to the open state (A2,R*). In principle, the receptor can undergo transitions to and from each of these states to several desensitized conformations (D1, D2). However, under the conditions of our experiments, with high ACh concentrations, we have made the simplifying assumption that the overall kinetics of ACh evoked current decay is dominated by desensitization from the liganded/open state. Moreover, to explain the biphasic time course of decay of ACh-evoked currents, we include two distinct desensitized states (A1,D1 and A1,D2). With these simplifying assumptions, analysis of the reaction scheme outlined in the brackets predicts that the ACh-evoked current decays according to the following equation

\[
A_2 R^*(x,t) - \sigma \frac{k_1}{\lambda} [1 - A_2 R^*(x,t)] e^{-\lambda t} + \lambda_1 [1 - A_2 R^*(x,t)] e^{-\lambda_2 t} + \lambda \lambda_1 \lambda_2 [1 - A_2 R^*(x,t)] e^{-\lambda t} + \lambda_2 [1 - A_2 R^*(x,t)] e^{-\lambda_1 t} + A_2 R^*(x,t)
\]

where \(A_2 R^*(x,t)\) is the number of active receptors after equilibrium desensitization has been reached and where
In our experiments we have measured three parameters of desensitization: \( \tau_1 \) (or \( \tau_2 \)) = 1/\( \lambda_1 \), \( \tau_2 \) (or \( \tau_3 \)) = 1/\( \lambda_2 \), and the relative amplitude of the fast and slow components of desensitization \( (A/B) \) given by

\[
A/B = \frac{k_3[1 - A_2R^*(\infty)]}{k_1[1 - A_1R^*(\infty)]} \quad (A4)
\]

With the use of the values for \( \tau_1 \), \( \tau_2 \) and \( A/B \) obtained with 100 \( \mu \)M ACh, we can solve directly for \( k_1 \). This yields a \( k_1(\text{ACh alone}) \) of 0.63 s\(^{-1}\). In the presence of SP, we obtain \( k_1(\text{ACh+SP}) \) of 0.81 s\(^{-1}\).

The kinetic scheme (Eq. 1) has four unknown parameters \( (k_{1-4}) \), whereas we have only three experimentally determined quantities \( \tau_1 \), \( \tau_2 \), and \( A/B \). However, if we make the additional simplifying assumption that \( k_3 \approx k_4 \) (which seems reasonable because, under the conditions of our experiments, virtually all AChRs are desensitized at equilibrium; i.e., \( A_2R^*(\infty) = 0 \) ), we can solve for \( k_3 \) and \( k_4 \) giving the following equations:

\[
l_1 + l_2 = k_1 + k_1 + k_3 + k_3 \approx k_1 + k_2 \quad (A5)
\]

\[
l_1l_3 = k_3l_3 + k_4l_4 + k_4l_4 \approx k_3l_1 \quad (A6)
\]

Solving these two equations for both \( k_3 \) and \( k_4 \) for ACh (100 \( \mu \)M) with and without SP reveals that the peptide alters both of these rate constants. Thus \( k_3(\text{ACh alone}) \) equals 0.421 s\(^{-1}\), \( k_3(\text{+SP}) \) equals 0.134 s\(^{-1}\), and \( k_4(\text{ACh alone}) \) equals 0.136 s\(^{-1}\), \( k_4(\text{+SP}) \) equals 0.296 s\(^{-1}\).

This analysis of the effect of SP indicates that SP causes 1) a small increase in the forward rate of desensitization to \( A_2D_1 \), increasing \( k_1 \) by \( \approx 25\% \); and 2) a more substantial increase \( \approx -60\% \) in the second forward rate constant, \( k_2 \), to the \( A_2D_2 \) desensitized state. 3) The most profound effect of SP is to slow the rate of recovery \( (k_2) \) from the intermediate desensitized state. \( A_2D_2 \). This is consistent with the observed decrease in the rate of recovery from early phases of desensitization (Fig. 3B). Overall, this supports the notion that SP enhances AChR desensitization, both accelerating the transition to, and slowing the recovery from, the desensitized states.

This model also predicts the fraction of receptors that would be expected to reside in the \( D_2 \) state after a 10-s application of 100 \( \mu \)M ACh. In the absence of SP, this fraction is predicted to be 0.5, whereas, in the presence of SP, the predicted fraction residing in \( D_2 \) increases to 0.88. The data in Fig. 4 show that, with repeated applications of ACh, there is a progressive decline in the peak ACh-evoked response, reflecting a very slow recovery from a desensitized state. One possibility is that occupancy of the \( D_2 \) state might be related to this long-lived desensitized state. Examination of the actual reduction in the peak ACh response following the first ACh application reveals that the second response is reduced by 40% relative to the first. This is similar to the predicted value of 50% occupancy of the \( D_2 \) state. The small discrepancy could reflect a very slow recovery from the \( D_2 \) state during the time between ACh applications with a time constant of \( \approx 1,300 \) s. In the presence of SP, the discrepancy between the predicted occupancy of the \( D_2 \) state and the reduction in the size of the second peak ACh response (88% predicted, 60% measured) could be explained if SP sped the rate of recovery from the very slow desensitized state from 1,300 to \( \approx 800 \) s\(^{-1}\).

In summary, this analysis suggests that SP enhances the rate of transitions to both the \( D_1 \) and \( D_2 \) desensitized states, slows the recovery from the \( D_1 \) to the open state, and may enhance recovery from the \( D_2 \) state. The net effect of the peptide, which are summarized in Table 2, are to enhance the rate of AChR desensitization and to stabilize the desensitized conformation.

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