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Characterization of the Binding of [^{3}H]Substance P to the Nicotinic Acetylcholine Receptor of *Torpedo* Electroplaque

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SUMMARY

The binding of [^{3}H]substance P to nicotinic acetylcholine receptor-enriched *Torpedo* electroplaque membranes was characterized. In the absence of cholinergic agonist, [^{3}H]substance P binding was displaced by unlabeled substance P with an IC_{50} of 31 ± 7 μM. In the presence of 10 mM carbamylcholine, displacement reflected two populations of binding sites with IC_{50} values of 0.93 ± 0.39 and 30 ± 5 μM, with the higher affinity component contributing 69 ± 2% of the inhibition. Equilibrium binding parameters were calculated by transformation of the concentration dependences of inhibition into saturation isotherms. In the absence of agonist, substance P bound with a K_d of 42 ± 7 μM to 3–4 sites/α-bungarotoxin binding site. In the presence of agonist, substance P bound to two sites, a low affinity site not significantly different from that seen in the absence of agonist (K_d = 25 ± 8 μM, ~3 sites/α-bungarotoxin site) and a high affinity site with a K_d of 0.55 ± 0.32 μM (approximately 1 site/2 α-bungarotoxin sites, 1 site/receptor). The increase in substance P binding induced by carbamylcholine was blocked by the nicotinic antagonists α-bungarotoxin and *d*-tubocurarine but was not affected by the muscarinic antagonist atropine. The concentration dependence of the carbamylcholine-induced increase had two components, with EC_{50} values for the agonist of 9.1 ± 4.2 μM (56 ± 16% of the increase) and 1.3 ± 0.5 mM. The structural specificity of agonist-dependent high affinity substance P binding was identical to that seen for inhibition of nicotinic receptor activation and substantially different from that of binding to the G protein-coupled tachykinin receptors. From the time courses of association, it appears that substance P binds preferentially to a transient agonist-induced receptor state. The γ and δ subunits of the receptor were specifically labeled in an agonist-dependent manner after cross-linking of [^{3}H]substance P to the receptor with the bifunctional cross-linking reagent bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone or after photoaffinity labeling of the receptor with ^{125}I-*p*-benzoylphenylalanine-substance P. These results demonstrate the existence of a high affinity agonist-induced binding site for substance P on the nicotinic acetylcholine receptor that probably mediates the noncompetitive inhibition by the peptide of receptor activation.

SP is an 11-amino acid peptide that functions as a neuronally released transmitter in the central and peripheral nervous systems, where it is widely but selectively distributed (1). In many systems, SP does not act as a neurotransmitter in the conventional sense but acts as a neuromodulator, altering the excitability or responsiveness of the postsynaptic membrane to other transmitters (2). Modulation by SP of the postsynaptic responsiveness of nAChRs has been demonstrated in several systems, where the peptide has been shown to inhibit receptor activation noncompetitively. Inhibition of nAChR activation by SP was first described by Steinacker and Highstein (3) for the Mauther fiber-giant fiber synapse of the hatchet fish. Since this initial report SP has been shown to attenuate nAChR responses in numerous neuronal and muscle systems, including spinal Renshaw cells (4), adrenal chromaffin cells (5), sympathetic ganglia and skeletal muscle endplates (6), the neuronal cell line PC12 (7), the muscle cell line BC_3H1 (8), and recently, in our laboratory, *Torpedo* electroplaque membrane vesicles (9). The structural specificity of inhibition of nAChR activation by SP in these systems (8–11) clearly demonstrates that it is not mediated by any of the G protein-coupled tachykinin receptor subtypes (12).

There is significant evidence for a physiological role for modulation of nAChR responsiveness by SP. In the central nervous system SP has been found in neurons that synthesize acetylcholine (13). In the periphery SP-containing fibers have been identified in sympathetic ganglia (14) and recently presynaptically at the neuromuscular junction (15). However, the strongest evidence for a physiological role for SP modulation of nAChRs is in the adrenal gland, where the peptide is present in nerves innervating the organ (16), is released upon stimula-

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ABBREVIATIONS: SP, substance P; αBgt, α-bungarotoxin; BSCOES, bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone; Carb, carbamylcholine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; nAChR, nicotinic acetylcholine receptor; [Phe^6(*p*Bz)]SP, *p*-benzoylphenylalanine-substance P; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PCP, phencyclidine.

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tion of the splanchnic nerve (17), and affects catecholamine secretion *in vitro* and *in vivo* (18).

The most extensive studies of the mechanism of inhibition by SP of nAChR activation had previously been done with neuronal adrenal gland-derived cells, i.e., isolated adrenal chromaffin cells (5, 10, 19, 20) and the rat adrenal tumor-derived cell line PC12 (7, 8, 11, 21). However, when it became apparent that SP inhibited muscle-type as well as neuronal receptors, we decided to use *Torpedo* electroplaque membranes as a model system to study the mechanism of action of SP on the nAChR. Because of the large amount of highly purified receptor that can be obtained, this system allows detailed biochemical studies that cannot be done in other systems. SP was found to noncompetitively inhibit Carb-stimulated $^{22}Na^+$ efflux from *Torpedo* membrane vesicles in a manner consistent with what was observed in other systems (9). We demonstrated that SP allosterically modulates the binding of the noncompetitive inhibitor PCP (22, 23) and of nAChR agonists (23), indicating a direct interaction with the receptor that is not mediated by the high affinity local anesthetic or cholinergic agonist binding sites. The studies described here examine directly the interaction of SP with the nAChR by characterization of the binding of [^{3}H]SP to the nAChR of *Torpedo* electroplaque membranes.

Experimental Procedures

Materials. [^{3}H]SP (30–40 Ci/mmol) and ^{125}I-αBgt (70–140 Ci/mmol) were purchased from New England Nuclear (Boston, MA). ^{125}I-[Phe^8(*p*Bz)]SP (~2000 Ci/mmol) was a generous gift of Dr. Norman Boyd (University of Massachusetts Medical Center). Unlabeled SP and related peptides were obtained from Bachem (Torrance, CA). BSCOES was purchased from Pierce Chemical Co. (Rockford, IL). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Membrane preparation. nAChR-enriched membranes (~2 nmol of αBgt sites/mg of protein) were prepared from frozen *Torpedo californica* electroplaques (Biofish Associates, Georgetown, MA), as described by Oswald (24). The membranes were aliquoted and stored in liquid nitrogen in *Torpedo* phosphate saline (in mM: 250 NaCl, 5 KCl, 3 $CaCl_2$, 2 $MgCl_2$, 5 $NaPO_4$, pH 7.4), at a concentration of 8–20 μM αBgt sites. The concentration of αBgt sites was determined with the DE81 filter disk assay of Klett *et al.* (25), measuring the equilibrium binding of ^{125}I-αBgt in 0.1% (w/v) Triton X-100, 10 mM Tris, pH 7.4. Protein concentrations were determined by the method of Bradford (26), with bovine serum albumin as the standard.

[^{3}H]SP binding assay. Membranes (0.35 μM αBgt sites) were incubated with 250–400 nM [^{3}H]SP (0.5–2 Ci/mmol, isotopically diluted with unlabeled peptide) at room temperature under the appropriate conditions, in 200 μl of 150 mM NaCl, 1 mM EGTA, 0.1 mg/ml bovine serum albumin, 50 mM MOPS-NaOH, pH 7.4. For most measurements of equilibrium binding, bound and free radioligands were separated after incubation for 10–20 min, by centrifugation at 10,000 × *g* for 10 min in an Eppendorf microfuge. The pellets were collected as described previously (22), and radioactivity was determined by scintillation counting. The concentration of free [^{3}H]SP was determined directly from an aliquot (50 μl) of the centrifugation supernatant. In some cases (Figs. 4 and 5) bound and free radioligands were separated by rapid vacuum filtration through no. 32 glass fiber filters (Schleicher & Schuell, Keene, NH) that had been presoaked in 0.5% polyethylenimine in binding buffer, followed by three rapid rinses with 2 ml of buffer. The only significant differences between binding measured by filtration and that measured by centrifugation were a decrease in nonspecific binding and a reduction of the low affinity component in filtration measurements. Saturation isotherms were generated from the concentration dependence of the inhibition of [^{3}H]SP binding by unlabeled SP by calculating the specific activity at each total concentration of peptide. It should be noted that the preferred method of performing saturation isotherms is to measure specific binding at increasing concentrations of radioligand with a constant specific activity. For economic reasons this was not possible for these experiments, and saturation isotherms were measured indirectly. The increased error inherent in this method because of the low fractional occupancy was at least partially compensated for by the high concentrations of receptor that could be used. The time courses of [^{3}H]SP binding were determined by rapid separation of bound and free radioligand by filtration. Association kinetics were determined by measuring binding at increasing times after the addition of [^{3}H]SP under three conditions, 1) no Carb, i.e., [^{3}H]SP added to the incubation in the absence of Carb; 2) prior addition, i.e., 10 mM Carb added to the membranes 10 min before the addition of [^{3}H]SP; and 3) simultaneous addition, i.e., 10 mM Carb and [^{3}H]SP added together at time zero. Dissociation kinetics were determined after prior equilibration of the membranes with [^{3}H]SP in the presence or absence of 10 mM Carb, with initiation of dissociation at time 0 by the addition of 560 μM unlabeled SP. For all binding assays nonspecific binding was determined in the presence of 560 μM SP.

Covalent labeling with [^{3}H]SP. Covalent labeling of the nAChR by [^{3}H]SP with the bifunctional cross-linking reagent BSCOES was done following the method of Howard *et al.* (27) for covalent labeling of opioid receptors by β-endorphin. Membranes were diluted to a concentration of 1 μM αBgt sites in 60 μl of binding buffer and were incubated for 30 min at room temperature with 1 μM [^{3}H]SP (36 Ci/mmol) and 10 mM Carb or 100 μM unlabeled SP, as described in the legend to Fig. 6. After a 2-fold dilution in ice-cold buffer, the membranes were centrifuged at 10,000 × *g* for 10 min. The pellet was resuspended in 0.5 ml of buffer containing 10 mM Carb and 1 mM BSCOES. After 15 min at 4°, the reaction was terminated by 10-fold dilution with ice-cold buffer and centrifugation at 10,000 × *g* for 10 min. The resulting pellet was retained for electrophoresis as described below.

Photoaffinity labeling with ^{125}I-[Phe^8(*p*Bz)]SP. Photoaffinity labeling of the nAChR by ^{125}I-[Phe^8(*p*Bz)]SP was done essentially as described by Boyd *et al.* (28) for photoaffinity labeling of neurokinin receptors. Membranes (1–2 μM αBgt sites) were incubated for 10 min at room temperature in the dark with 5 nM ^{125}I-[Phe^8(*p*Bz)]SP and 10 mM Carb or 100 μM unlabeled SP, in 20 μl of *Torpedo* phosphate saline with 1 μl/ml protease inhibitor cocktail made up of (mg/ml, in dimethylsulfoxide) 174 phenylmethylsulfonyl fluoride, 198 1,10-phenanthroline, 185 iodoacetamide, 1 antipain, 1 leupeptin, and 1 pepstatin. The membranes were irradiated for 5–10 min in polystyrene tissue culture dishes at a distance of 13 cm, with a 100-W long-wavelength (354-nm) UV lamp (model R-526; Ultra-Violet Products, Inc.).

SDS-PAGE and autoradiography. Photoaffinity-labeled membranes or BSCOES-cross-linked membrane pellets were solubilized in SDS-PAGE sample buffer (5% β-mercaptoethanol, 10% glycerol, 2% SDS, 60 mM Tris·HCl, pH 6.8). Samples (100 μg of protein) were electrophoresed in 10% acrylamide slab gels according to the method of Laemmli (29). Gels were stained with Coomassie blue, dried at 80° for 3 hr on a gel drier (Hoefer Scientific Instruments, San Francisco, CA), and exposed at −80° to Kodak X-OMAT film with two intensifying screens. [^{3}H]SP-labeled receptor gels were soaked in En^{3}Hance (New England Nuclear) for 30 min before drying. Two-day exposures were sufficient for membranes labeled with ^{125}I-[Phe^8(*p*Bz)]SP; 2–3-month exposures were required to visualize labeling with [^{3}H]SP/BSCOES. The molecular weights of the radiolabeled bands were determined with Bio-Rad low molecular weight standards. To quantitate the covalent incorporation of [^{3}H]SP into receptor subunits, the four subunits identified by Coomassie staining were cut from the gel, dissolved in 30% H_2O_2 overnight at 60°, and scintillation counted.

Data analysis and statistics. Data were analyzed by nonlinear least squares fitting with KaleidaGraph (Synergy Software, Reading, PA) on a Macintosh II or SE/30 computer. The concentration dependences of the inhibition of specific [^{3}H]SP binding were fit with the equation $B = B_1\{IC_{501}/(IC_{501} + [I])\} + B_2\{IC_{502}/(IC_{502} + [I])\}$, with $B_2 = 0$ for fitting with a single site. B is radioligand bound in the presence of inhibitor at [I]; B_1 and B_2 are the bound components, inhibited by 50% when [I] equals IC_{501} and IC_{502}, respectively. Saturation isotherms

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generated from the inhibition of [³H]SP binding by unlabeled SP were fit with the equation $B = B_{max1}\{[SP]/([SP] + K_{d1})\} + B_{max2}\{[SP]/([SP] + K_{d2})\}$, with $B_{max2} = 0$ for fitting with a single site. B is the concentration of SP bound at the free concentration [SP]; B_{max1} and B_{max2} are the concentrations of binding sites with equilibrium dissociation constants K_{d1} and K_{d2}, respectively. Saturation data were plotted by the method of Scatchard (30). The concentration dependence of the Carb-induced increase in [³H]SP binding was fit with the equation $B = B_o + B_{inc1}\{[Carb]/([Carb] + EC_{501})\} + B_{inc2}\{[Carb]/([Carb] + EC_{502})\}$, where B is radioligand bound in the presence of [Carb], B_o is radioligand bound in the absence of Carb (control), and B_{inc1} and B_{inc2} are the components increased by 50% when [Carb] equals EC_{501} and EC_{502}, respectively. Association time courses were fit with the equation $B = B_{e1}(1 - e^{-k_1 t}) + B_{e2}(1 - e^{-k_2 t})$, with $B_{e2} = 0$ for fitting with a single component. B is the radioligand bound at time t; B_{e1} and B_{e2} are the components that bind with apparent rate constants k_1 and k_2, respectively. Dissociation time courses were fit with the equation $B = B_o(e^{-kt})$, where B_o is the radioligand bound at time 0 and k is the apparent rate constant of dissociation. Significance of the improvement of the fit for two components versus one was determined by the F ratio test (31). Data are expressed as means ± standard errors and statistical significance was assessed by Student's t test.

Results

In the absence of the nAChR agonist Carb, the binding of [³H]SP to *Torpedo* membranes was inhibited by unlabeled SP in a manner consistent with an interaction with a single population of low affinity sites (IC_{50} = 31 ± 7 μM, eight experiments) (Fig. 1A). As seen in the Scatchard plot in Fig. 1B, saturation isotherms generated from the inhibition curves showed a relatively high concentration of low affinity sites (Table 1). The concentration of these low affinity sites varied significantly between membrane preparations; however, in all cases it was >2-fold greater than the concentration of αBgt sites. In the presence of 10 mM Carb, specific binding of [³H]SP increased 3–5-fold (Fig. 1A; see also Fig. 2) and inhibition by unlabeled SP was biphasic ($p < 0.05$, F ratio test), with IC_{50} values of 0.93 ± 0.39 and 30 ± 5 μM and with the higher affinity component making up 69 ± 2% of the displacement (eight experiments). The IC_{50} value of the low affinity component and that for inhibition in the absence of Carb were not significantly different. Saturation isotherms in the presence of Carb were also, of course, biphasic (Fig. 1B), with submicromolar K_d and B_{max} values for the higher affinity component and K_d and B_{max} values for the lower affinity component not significantly different from those measured in the absence of Carb (Table 1). The number of Carb-induced high affinity sites was approximately equal to one half the number of αBgt binding sites.

The increase in [³H]SP binding induced by Carb was mediated by an interaction of the agonist with the nAChR, because it was completely inhibited by the nAChR antagonists αBgt and *d*-tubocurarine and not affected by the muscarinic antagonist atropine (Fig. 2). The nAChR antagonists had no effect on [³H]SP binding in the absence of Carb. The concentration dependence of the Carb-induced increase in [³H]SP binding is shown in Fig. 3. Unexpectedly, two components to the increase were observed, with EC_{50} values of 9.1 ± 4.2 μM (56 ± 16% of the increase) and 1.3 ± 0.5 mM (three experiments). The increase thus appears to be meditated by two independent binding sites for Carb. The lower EC_{50} is consistent with an interaction with the agonist/activation site of the receptor; the higher EC_{50} would appear to reflect an interaction with a regulatory low affinity agonist binding site, as described by Takeyasu *et al.* (32).

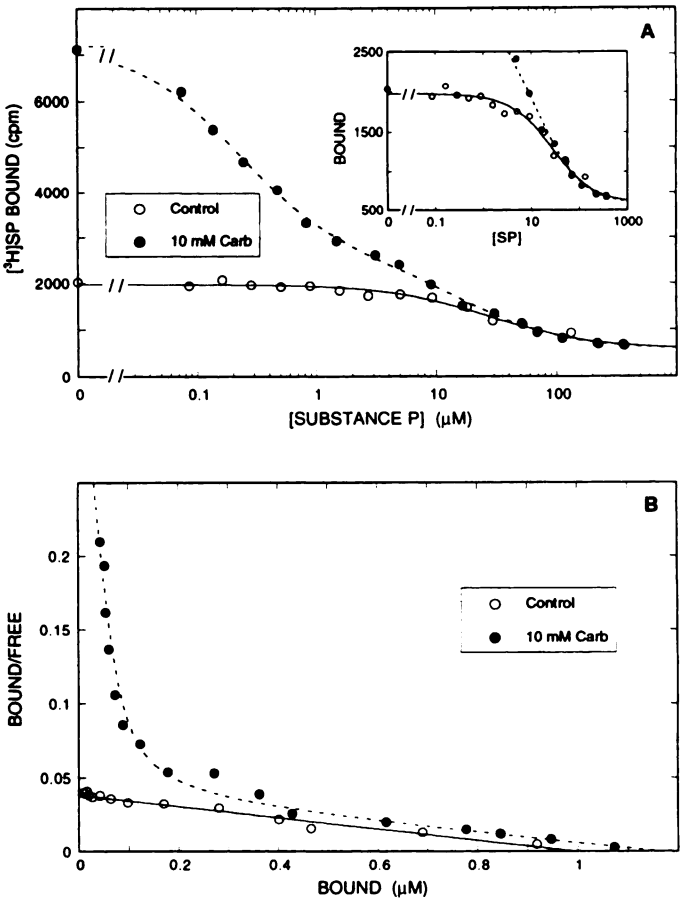

Fig. 1. Concentration dependence of inhibition of [³H]SP binding by unlabeled SP in the absence and presence of 10 mM Carb (A) and Scatchard plots of saturation isotherms generated from the inhibition curves (B). Equilibrium binding of 265 nM [³H]SP to *Torpedo* membranes (0.35 μM αBgt sites) was measured by centrifugation assay with increasing concentrations of unlabeled SP in the absence (○) and presence (●) of 10 mM Carb. A, Total bound counts are plotted as a function of the free concentration of SP. *Inset*, same data on an expanded scale. *Lines* are from nonlinear fits, as described in Experimental Procedures. In the absence of Carb the IC_{50} was 29 μM (B_{max} = 1339 cpm); in the presence of Carb there were two components, with IC_{50} values of 20 and 0.31 μM (B_{max} = 1758 and 4790 cpm, respectively). Nonspecific binding was 715 cpm. B, Saturation isotherms were generated from inhibition data and are shown as Scatchard plots. *Lines* are from nonlinear fits to the saturation data. In the absence of Carb the K_d and B_{max} values were 26 and 1.0 μM, respectively; in the presence of Carb the K_d and B_{max} values were 30 and 1.1 μM for the low affinity component and 0.48 and 0.10 μM for the high affinity component. In the presence of Carb fits were significantly improved for two sites versus one ($p < 0.05$, F ratio test). *Data points* are from a representative experiment done eight times in duplicate (see Table 1).

The structural specificity of high affinity [³H]SP binding was determined by measuring the concentration dependence of inhibition of binding by several SP-related peptides in the presence of 10 mM Carb (Fig. 4). Although the structure-activity profile for inhibition of nAChR activation shows some similarities to that of the tachykinin receptors (sensitivity to carboxyl-terminal deletions and relative insensitivity to amino-terminal deletions), substantial differences with respect to the potency of the nonmammalian tachykinins physalaemin, eledoisin, and kassinin were observed (8–11) (see Table 2). As had been reported for inhibition of receptor activation (8–11), binding was dramatically dependent on the carboxyl-terminal portion of the peptide, amino-terminal deletions caused only incremen-

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TABLE 1

Equilibrium constants for SP binding to *Torpedo* membranes in the absence and presence of Carb

The equilibrium dissociation constants (K_d) and concentrations of binding sites (B_{max}) for SP were determined in the absence and presence of 10 mM Carb, as described for Fig 1. The concentration of αBgt binding sites in the assay was 0.35 μM. Values are means ± standard errors from eight experiments done in duplicate.

Carb	Binding affinity[a]	K_d	B_{max}
		μM	μM
None	Low	42 ± 9	1.2 ± 0.6
10 mM	Low	25 ± 8	0.9 ± 0.6
	High	0.55 ± 0.32	0.15 ± 0.05

[a] In the absence of Carb no high affinity sites were observed; in the presence of Carb fits were significantly improved with a two-site model ($p < 0.05$, F ratio test).

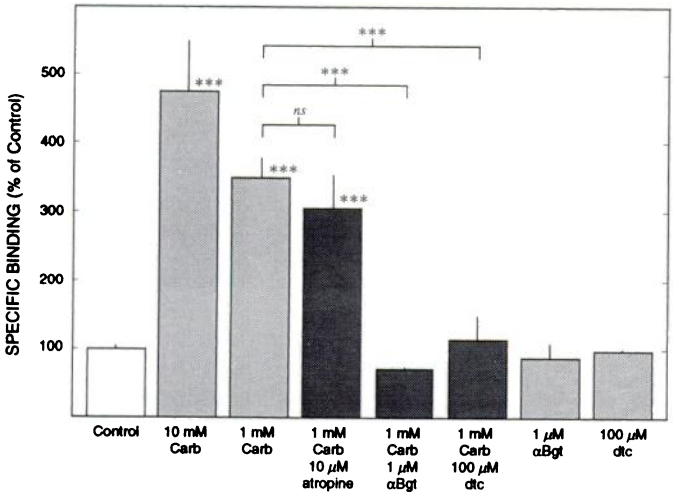

Fig. 2. Effects of cholinergic ligands on [^{3}H]SP binding. Equilibrium binding of 300 nM [^{3}H]SP was measured by centrifugation assay. Nonspecific binding determined in the presence of 560 μM SP was subtracted and specific binding has been normalized to binding observed in the absence of added ligands (*Control*). Incubations were done in the presence of 1 or 10 mM Carb, 1 mM Carb plus 10 μM atropine, 1 mM Carb plus 1 μM αBgt, 1 mM Carb plus 100 μM *d*-tubocurarine (*dtc*), 1 μM αBgt alone, or 100 μM *d*-tubocurarine alone. Values are means ± standard errors from three to nine determinations done in duplicate. *Asterisks on the bars*, significance compared with control; *brackets*, other statistical comparisons. ***, $p < 0.001$; *ns*, $p > 0.1$.

tal decreases in affinity, and the structurally related nonmammalian tachykinins physalaemin, eledoisin, and kassinin had essentially no effect (Table 2). Although a filtration assay was used to minimize the contribution of the low affinity component and fits to the inhibition curves were not improved using a two-site model ($p > 0.1$, F ratio test), there was still some contribution by the low affinity component apparent from the somewhat higher IC_{50} value for SP in the filtration assay, compared with that of the high affinity component measured with the centrifugation assay (3.0 versus 0.9 μM).

The time courses of association and dissociation of [^{3}H]SP are shown in Fig. 5. In the absence of Carb, 0.4 μM [^{3}H]SP associated with and dissociated from the low affinity site with $t_{1/2}$ values of ~30 and <15 sec, respectively (Fig. 5). Based on results from studies of the association kinetics of the noncompetitive inhibitors PCP (33, 34) and chlorpromazine (35), we examined the kinetics of [^{3}H]SP binding in the presence of Carb under two conditions, i.e., after prior equilibration of the membranes with Carb (when the receptor is primarily in the desensitized state) and with simultaneous addition of [^{3}H]SP and Carb (when the receptor is transiently in the open and rapidly desensitized states). The association time courses were

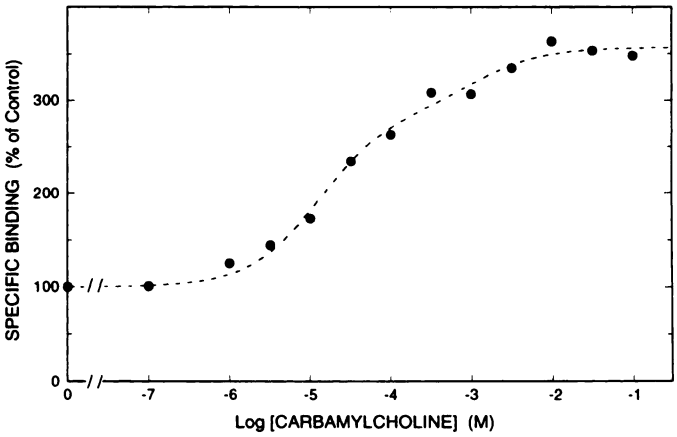

Fig. 3. Concentration dependence of the Carb-induced increase in [^{3}H]SP binding. Equilibrium binding of 300 nM [^{3}H]SP was measured by centrifugation assay in the presence of increasing concentrations of Carb. Nonspecific binding determined in the presence of 560 μM SP was subtracted and specific binding has been normalized to binding observed in the absence of Carb. *Line* is from a nonlinear fit: the increase was biphasic ($p < 0.05$, F ratio test), with EC_{50} values of 13 μM and 1.1 mM. The higher affinity component contributed 72% of the total increase (B_{inc1} = 185%, B_{inc2} = 71%). *Data points* are from a representative experiment done three times in duplicate (see text).

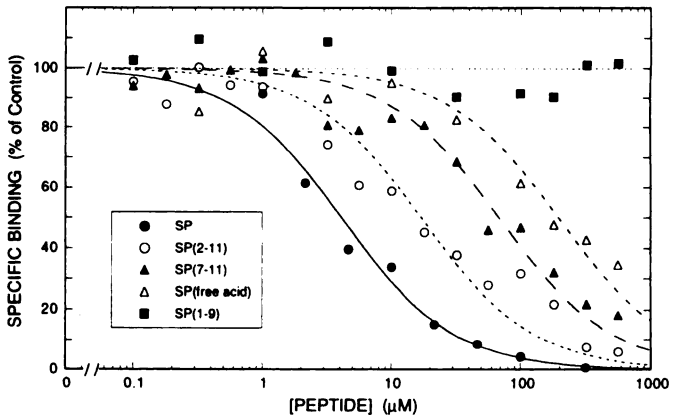

Fig. 4. Concentration dependence of inhibition of [^{3}H]SP binding by SP and related peptides. Equilibrium binding of 275 nM [^{3}H]SP in the presence of 10 mM Carb with increasing concentrations of various peptides was measured by filtration assay. Nonspecific binding was subtracted and specific binding has been normalized to binding observed in the absence of added ligands (100%). *Lines* are from nonlinear fits for a single population of sites. IC_{50} values were as follows: SP, 6.3 μM; SP_{2-11}, 17 μM; SP_{7-11}, 68 μM; $SP_{free\ acid}$, 197 μM; SP_{1-9}, ≫1 mM. *Data points* are from a representative experiment done three times in duplicate (see Table 2).

very different under the two conditions and the results were similar to those described previously for PCP and chlorpromazine. After prior equilibration with Carb, association was biphasic, with a rapid component ($t_{1/2}$, ~30 sec) that appears to reflect binding to the low affinity component and is similar in apparent rate and amplitude to that seen in the absence of Carb and a slow component ($t_{1/2}$, ~4 min) that is due to binding to the high affinity site on the desensitized receptor (Fig. 5A). When [^{3}H]SP and Carb were added simultaneously, binding equilibrated almost instantaneously (Fig. 5A). We interpret the increased rate of binding under these conditions as indicating that SP binds extremely rapidly to a transient agonist-induced state, either the open state or the rapidly desensitized state, as previously proposed for other noncompetitive inhibitors (34,

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TABLE 2

IC_{50} values for SP and related peptides for inhibition of [³H]SP binding and of Carb-stimulated $^{22}Na^+$ influx

IC_{50} values for SP and structurally related peptides were determined in the presence of 10 mM Carb using a filtration assay, as described in Fig. 4. Values are means ± standard errors from three experiments done in duplicate.

Peptide	IC_{50}		
	[³H]SP binding (*Torpedo* membranes)	$^{22}Na^+$ influx (BC_3H1 cells)[a]	$^{22}Na^+$ influx (PC12 cells)[a]
		μM	
SP	3.0 ± 1.2	8.2 ± 4.6	1.2 ± 0.1
SP_{2-11}	9.2 ± 7	36.6 ± 4.4	2.9 ± 0.2
SP_{7-11}	77 ± 9	13.9 ± 2.4	13.3 ± 0.8
$SP_{free\ acid}$	196 ± 72		35.9 ± 3.9
SP_{1-9}	>1000[b]	>100	>100
SP_{1-4}	>1000[b]	>100	>100
Physalaemin	>1000[b]	≈100	>100
Eledoisin	>1000[b]	>100	>100
Kassinin	>1000[b]		>100

[a] IC_{50} values are from Simasko *et al.* (8), for $^{22}Na^+$ influx measured in the presence of 1 mM Carb.
[b] No inhibition was observed at the highest concentration used (560 μM).

35). After equilibration in the presence of Carb, [³H]SP dissociation was exponential and relatively slow, with a $t_{1/2}$ of ~2 min (Fig. 5B).

To identify with which receptor subunit(s) SP was interacting, two affinity labeling methods were used, 1) direct cross-linking of [³H]SP to the receptor with the bifunctional reagent BSCOES, as described by Howard *et al.* (27) for the labeling of opioid receptors, and 2) photoaffinity labeling with ^{125}I-[Phe⁸(*p*Bz)]SP, as described by Boyd *et al.* (28) for the labeling of neurokinin receptors. To assess specific binding of the subunits, labeling reactions were done in the absence and presence of 100 μM unlabeled SP. After BSCOES-cross-linking of [³H]SP to the receptor, no specific labeling of the α or β subunits was observed even in the presence of agonist, as assessed by the difference seen in the absence and presence of 100 μM SP (Fig. 6). The δ subunit was specifically labeled, as seen by both counting of gel slices (Fig. 6A) and autoradiography (Fig. 6B). Specific labeling of the γ subunit was lower and not always apparent in the autoradiographs (Fig. 6). In the presence of Carb, specific labeling of the γ and δ subunits increased significantly. The increase was also blocked by αBgt (data not shown). Similar results were obtained using the photoaffinity label ^{125}I-[Phe⁸(*p*Bz)]SP (Fig. 7). No specific labeling of the α, β, or γ subunits was observed, even in the presence of agonist. Specific labeling of the δ subunit was seen only in the presence of Carb. The apparent increase in the labeling of the α and γ subunits in Fig. 7, *lane 2*, was due to unequal loading of the lanes and was not reproducible. It should be noted that with both methods there was no evidence for the presence of the SP tachykinin (neurokinin type 1) receptor, which would have been indicated by a labeled band of ~50 kDa (28). These results provide additional evidence that SP interacts directly with the nAChR and indicate that the δ (and perhaps γ) subunit contributes at least part of the high affinity binding site.

Discussion

These studies demonstrate that SP interacts directly and specifically with two populations of binding sites on the nAChR of *Torpedo* electroplaque. The lower affinity sites, of which there are several for each receptor, would appear to reflect nonspecific interactions either with the receptor, perhaps at

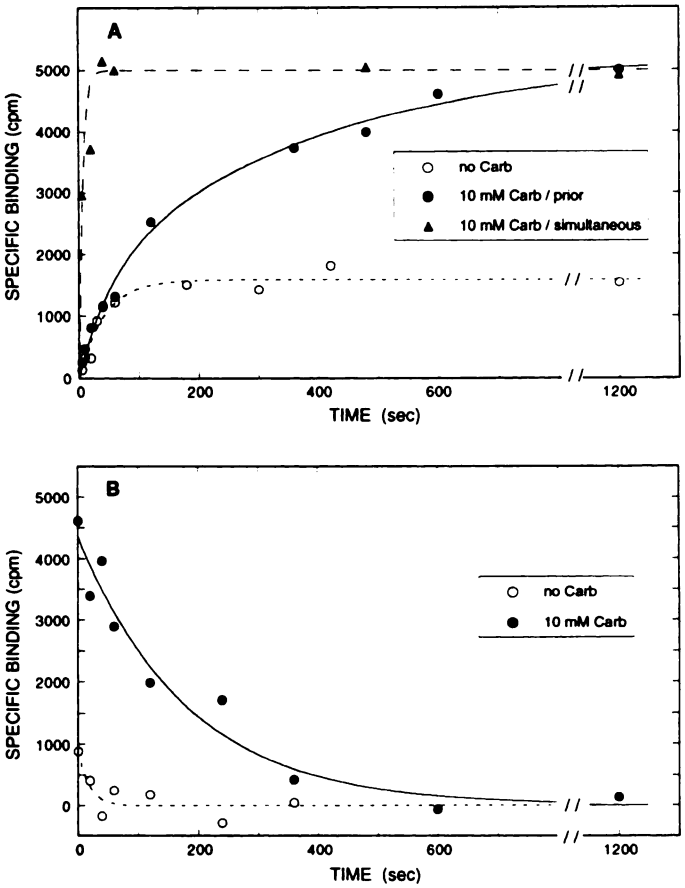

Fig. 5. Time courses of association (A) and dissociation (B) of [³H]SP binding in the absence and presence of 10 mM Carb. Binding of 400 nM [³H]SP was measured by rapid filtration. Nonspecific binding determined in the presence of 560 μM SP was subtracted. A, Association. [³H]SP was added to the membranes at time 0 under three different conditions, 1) in the absence of Carb (○), 2) in the presence of 10 mM Carb added 10 min before the addition of [³H]SP (●), and 3) in the presence of 10 mM Carb added simultaneously with [³H]SP (▲). *Lines* are from nonlinear fits; the line for condition 1 was fit by a single-exponential function with k = 0.024 sec^{-1} (B_e = 1592 cpm), the line for condition 2 was biexponential ($p < 0.05$, F ratio test), with k_1 = 0.020 sec^{-1} (B_{e1} = 1493 cpm) and k_2 = 0.0027 sec^{-1} (B_{e2} = 3693 cpm), and binding in condition 3 was too rapid to quantitate kinetically (B_e = 5000 cpm). B, Dissociation. Membranes were incubated for 10 min with [³H]SP in the absence (○) or presence of 10 mM Carb (●) before dissociation was initiated by the addition of 560 μM SP at time 0. Points at time 0 were measured before addition of unlabeled SP. *Lines* are from nonlinear fits to single exponentials: no Carb, k = 0.055 sec^{-1} and B_e = 902 cpm; 10 mM Carb, k = 0.0055 sec^{-1} and B_e = 4346 cpm. *Data points* are from a representative experiment done three times in duplicate.

the protein-lipid interface, as proposed for the low affinity site(s) of local anesthetic-type blockers (36), or with other components of the membrane. The high affinity component of binding, which is seen only in the presence of a cholinergic agonist and of which there is one site for each receptor, most likely represents the functionally relevant interaction with the receptor. The apparent equilibrium constant (Table 1) and structural specificity (Table 2) are similar to those for inhibition of receptor activation (8, 9) and for allosteric effects on the binding of various ligands to the receptor (22, 23). This agonist-induced high affinity SP binding reflects a unique binding site, because the structural specificity of inhibition (Fig. 4; Table 2) is significantly different from that of SP binding to the G protein-coupled tachykinin receptors. For each of the tachykinin receptor subtypes at least one of the

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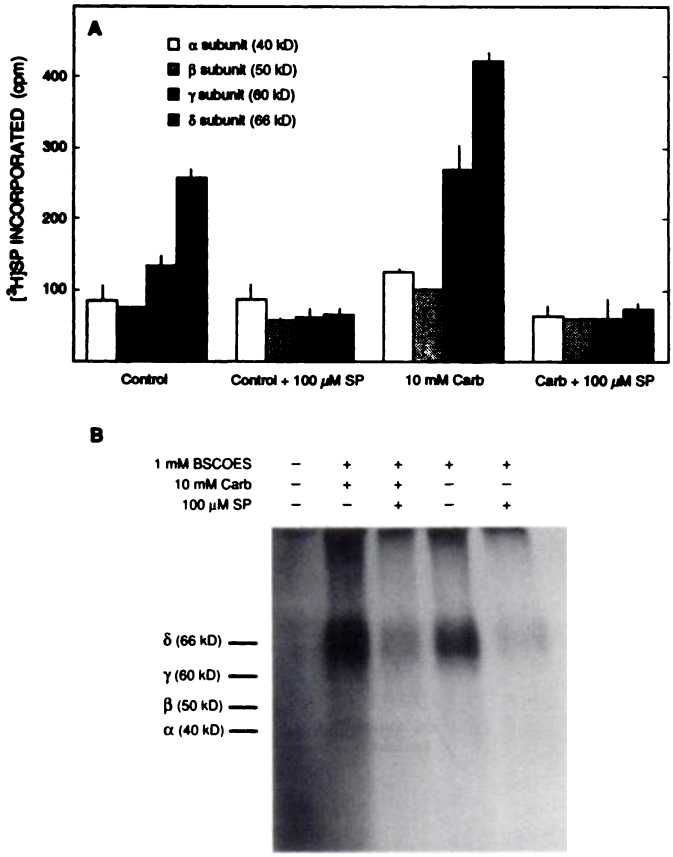

Fig. 6. Chemical cross-linking of [^{3}H]SP to nAChR subunits by BSCOES. Membranes (1 μM αBgt sites) were covalently labeled with 1 μM [^{3}H]SP by incubation with 1 mM BSCOES in the absence (*Control*) or presence of 10 mM Carb and/or 100 μM unlabeled SP as noted, and receptor subunits were separated by SDS-PAGE. A, Quantitation of [^{3}H]SP incorporation by scintillation counting. Subunits were identified by Coomassie blue staining and cut from the gel, and the incorporated radioactivity was determined. *Error bars*, standard errors from scintillation counting of each gel slice three times. B, Autoradiograph of [^{3}H]SP incorporation. Cross-linking was done as described above, with reaction mixtures as noted. Subunit bands were identified by Coomassie blue staining and the gel was subjected to autoradiography. Positions of the receptor subunits, identified by staining, are marked. Data shown are from a representative experiment done three times.

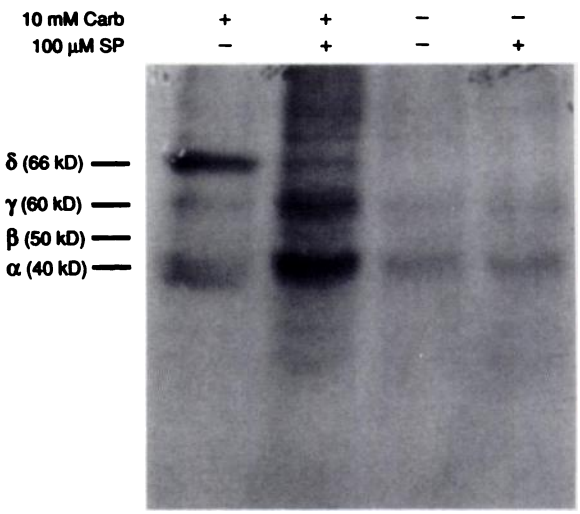

Fig. 7. Photoaffinity labeling of receptor subunits with ^{125}I-[Phe^8(*p*Bz)]SP. Membranes (1 μM αBgt sites) were photoaffinity labeled with 1 μM ^{125}I-[Phe^8(*p*Bz)]SP in the absence or presence of 10 mM Carb and 100 μM unlabeled SP as noted, and the receptor subunits were separated by SDS-PAGE. Subunit bands were identified by Coomassie blue staining and the gel was autoradiographed. Positions of the receptor subunits are marked. The autoradiograph is from a representative experiment done three times with similar results.

nonmammalian tachykinins physalaemin, eledoisin, or kassinin has an affinity equal to or greater than that of SP (12), and we observed no effect of any of these peptides on SP binding even at concentrations as high as 1 mM (Table 2).

The dependence of high affinity SP binding on Carb was mediated by an interaction with the nicotinic agonist binding site of the receptor. The nAChR antagonists αBgt and *d*-tubocurarine completely blocked the effect of Carb on SP binding, and the muscarinic antagonist atropine had no effect (Fig. 2). The concentration dependence of the effect of Carb on SP binding had two components, indicating that the increase is mediated by two independent binding sites for Carb (Fig. 3). The EC_{50} of the larger, high affinity component (9 μM) is consistent with an interaction with the agonist binding site (23). Agonist binding to this site, which mediates receptor activation and desensitization, would thus induce a conformational change in the receptor, allowing SP to bind with high affinity. In contrast, the lower affinity component (EC_{50}, ~1 mM) of the Carb-induced increase in SP binding was completely unexpected. It is unclear where Carb might be interacting to produce an effect at this low affinity. It is possible that the effect reflects an interaction with the low affinity agonist site described by Takeyasu *et al.* (32). This low affinity agonist site mediates an inhibition of channel activation that is voltage dependent and may be due to agonist binding within the channel pore, directly blocking ion flux. It appears that the binding of Carb to this low affinity site requires Carb binding to the higher affinity agonist binding site, because nAChR antagonists that block binding to this latter site completely inhibit the Carb-induced increase in SP binding (Fig. 2).

Although it is clear that different binding sites are involved (22, 23), there are several similarities between the high affinity binding of SP and that of the local anesthetic-type noncompetitive inhibitors such as PCP and chlorpromazine. For both SP and the local anesthetic-type inhibitors there is one site per receptor and its affinity is allosterically modulated by nAChR agonists (for review, see Ref. 37). In addition, as observed for PCP (34) and chlorpromazine (38), the rate of association of SP was much more rapid when agonist was added simultaneously with peptide than when the receptor was pre-equilibrated with agonist before addition (Fig. 5). This indicates rapid binding to a transient agonist-induced receptor state, such as the rapidly desensitized or open state. From rapid mixing measurements of photolabeling by [^{3}H]chlorpromazine, it appears that the local anesthetic-type inhibitors bind preferentially to the open state of the receptor (38). This may also be the case for SP.

Covalent affinity labeling by SP is also similar to that seen for the local anesthetic-type noncompetitive blockers. These agents can covalently label several of the nAChR subunits (for review, see Ref. 37) and in the case of chlorpromazine all of the subunits have been shown to be covalently labeled (38), as expected for a binding site within the ion channel pore. The agonist-dependent labeling of the γ and δ subunits by SP that we observed is also consistent with a binding site within the ion channel. SP labeling of the γ and δ subunits could not be due to a binding site at a subunit interface, as has been shown for *d*-tubocurarine (39), because the γ and δ subunits are not

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adjacent to each other in the receptor (40). The most likely way for two nonadjacent subunits to be labeled is from within the ion channel pore, where the subunits are in closest proximity. Verification of the location of the binding site will require identification of the amino acids labeled, to determine whether they are within the ion channel sequence of the receptor (the M2 segment). This has been done for several local anesthetic-type noncompetitive blockers, including chlorpromazine, and all of them covalently label amino acids within the M2 segment of the receptor (for review, see Ref. 37).

In summary, we have provided direct evidence that SP binds to the nAChR. The peptide binds to two sites, a low affinity site of which there are several per receptor and a high affinity, agonist-induced site of which there is one per receptor. Binding to the high affinity site is allosterically regulated by agonists. Binding to this site probably mediates inhibition of receptor activation and the allosteric effects on agonist and PCP binding to the receptor. This binding site may be within the ion channel pore of the receptor and more accessible when the receptor is in the open state than in the desensitized state.

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