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Effects of Substance P on the Binding of Ligands to Nicotinic Acetylcholine Receptors

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SUMMARY

The effect of substance P on the binding of many ligands that interact with the nicotinic acetylcholine receptor was examined in membrane preparations of *Torpedo* electroplaque and BC_3H-1 cells and in solubilized membranes of rat, chick, and goldfish brain. In the absence of carbamylcholine, the affinity of [^{3}H]phencyclidine for the high affinity local anesthetic binding site on *Torpedo* membranes was increased by substance P with an EC_{50} of approximately 5 μM. In the presence of carbamylcholine, which itself increases [^{3}H]phencyclidine binding affinity, substance P caused a decrease in the affinity of [^{3}H]phencyclidine. The concentration dependence of the inhibition, however, was inconsistent with a competitive interaction, since the apparent Hill coefficient was significantly less than one. We conclude from these results that substance P does not directly interact with the high affinity local anesthetic binding site on the nicotinic receptor of *Torpedo* membranes. Substance P also does not appear to interact directly with the agonist binding site since the peptide had no significant effect on [^{3}H]acetylcholine binding to *Torpedo* membranes. Substance P inhibited [^{125}I]α-bungarotoxin binding to both native and Triton X-100 solubilized *Torpedo* membranes, although the IC_{50} was 8-fold higher for the solubilized preparation (12 versus 93 μM). We interpret this inhibition in solubilized membranes as evidence that the peptide may interact directly with a binding site on the nicotinic acetylcholine receptor. Substance P also decreased the initial rate of [^{125}I]α-bungarotoxin to membranes prepared from BC_3H-1 cells (IC_{50} = 108 μM) and to solubilized membranes from rat, chick, and goldfish brain. In the brain membranes, however, the peptide did not completely inhibit binding; at the highest concentration examined (100 μM), the maximum inhibition observed was 60%. Consistent with the results for [^{3}H]acetylcholine binding to *Torpedo* membranes, the peptide had no effect on the binding of the cholinergic agonist [^{3}H](−)nicotine to these tissue preparations. These data suggest that substance P may have a general modulatory action on a subclass of nicotinic receptors that include muscle-type, ganglionic-type, and a putative subpopulation of central nervous system receptors.

Substance P is an unadecapeptide that has been shown to function as a neuronally released transmitter substance in many systems (1). In some cases the action of substance P, rather than consisting of a classical transmitter response of receptor activation, appears to reflect a modulatory role for the peptide (2). This modulation of postsynaptic responsiveness has been observed in several nAChR systems where the peptide has been shown to inhibit receptor activation noncompetitively. Although this effect was initially characterized in neuronal systems (3–11), it has more recently been reported for muscle-type nAChRs (11, 12).

The site at which substance P exerts its inhibitory action on nAChR activation appears to be different from that previously described for substance P action and may reflect a unique subtype of substance P receptor. The structurally related tachykinins physalaemin, eledoisin, and kassinin are much less potent than substance P in inhibiting nAChR-mediated catecholamine release in adrenal chromaffin cells (8) or $^{22}Na^+$ flux in PC12 cells (11). This pharmacology is different from that expected of either the SP-E or SP-P receptor subtypes previously described (13). In addition substance P antagonists do not block the effects of substance P but act as agonists, inhibiting nAChR responses (11, 14).

The mechanism of action for the noncompetitive inhibition of nAChR activation by substance P remains unclear. Results from biochemical studies of nAChR-mediated $^{22}Na^+$ flux in PC12 cells (10, 15) and electrophysiological patch-clamp studies of nAChR-mediated currents in adrenal chromaffin cells (8) have been inconclusive. The data are consistent with an enhancement of desensitization, as originally proposed by Stallcup and Patrick (10), as well as an allosteric block of nAChR

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ABBREVIATIONS: ACh, acetylcholine; αBgt, α-bungarotoxin; BSA, bovine serum albumin; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; HEPES, N-2-hydroxyethyl-piperazine-N′-2-ethanesulfonic acid; MOPS, 3-[N-morpholino]propanesulfonic acid; n_H, Hill coefficient; nAChR, nicotinic acetylcholine receptor; PCP, phencyclidine; Tris, tris(hydroxymethyl)aminomethane.

function that may indirectly enhance desensitization (8, 15). These mechanisms are very similar to those proposed for local anesthetic inhibition of nAChR activation (16), and similarities in the action of substance P and local anesthetics on single channel properties have recently been reported (17).

Because of the similarities with local anesthetic action and the fact that inhibition of nAChR function can be demonstrated for both neuronal and muscle-type nAChRs, a direct action of substance P on the nAChR appears probable. We have therefore examined the interaction of substance P with various binding sites on the nAChR, particularly the nAChR from *Torpedo* electroplaque. Purified *Torpedo* postsynaptic membrane fragments are highly enriched in nAChRs (20–50% of the protein), and many distinct binding sites have been characterized. Consequently this tissue provides an excellent model system for studying the interaction of substance P with the nAChR. There are three well-characterized classes of binding sites on the *Torpedo* nAChR: 1) the ACh site of which there are two per receptor monomer (for review see Ref. 18), 2) the site for the snake α-neurotoxins, such as αBgt, which at least partially overlaps with the acetylcholine binding site, and 3) the high affinity site for noncompetitive blockers such as local anesthetics, histrionicotoxin, and PCP. Using radioligand binding assays, we have examined the interaction of substance P with each of these sites in *Torpedo* membranes. In addition, the interaction of substance P with the αBgt binding site on BC_3H-1 membranes and with the αBgt and (−)nicotine binding sites on brain membranes was also investigated.

Experimental Procedures

Cell Culture

BC_3H-1 cells (19) were obtained from Dr. Palmer Taylor, University of California, San Diego (La Jolla, CA) and the American Type Culture Collection (Rockville, MD). Cells were grown and maintained on 100-mm plastic tissue culture plates in Dulbecco's modified Eagle's medium (4500 mg glucose/liter) with 10% (vol/vol) fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin as previously described (11). For the binding assays, cells were plated onto 30-mm plates and either allowed to grow to confluency for 2 weeks in high serum (20), or after 3 days the growth medium was replaced with medium containing 1% (vol/vol) fetal calf serum to induce differentiation and expression of nAChRs (21). Cells were harvested 5–14 days after the change to low serum medium, with medium changes every 3–4 days.

Tissue Preparation

Nicotinic acetylcholine receptor-rich membrane fragments were prepared from freshly dissected electric organ of *T. californica* or frozen organ of *T. nobiliana* by differential centrifugation by the method of Sobel *et al.* (22) as described by Oswald (23). The concentration of [^{125}I]αBgt binding sites was measured by the DE81 filter disc assay (24) using 1% (wt/vol) Triton X-100 in 10 mM Tris (pH 7.4). Membranes were stored until use in liquid nitrogen at a concentration of 8–20 μM (expressed in αBgt binding sites). To prepare solubilized *Torpedo* nAChRs, membranes were incubated in 1.0% Triton X-100 (50 mM MOPS/1 mM EGTA, pH 7.5) for 20 min at room temperature, and membranes were separated from solubilized receptors by centrifugation at 100,000 × *g* for 1 hr. The supernatant, containing the soluble nAChRs, was used immediately.

For preparation of BC_3H-1 membranes, differentiated cells were lysed by incubation on the tissue culture plates for 30 min at 4°C in 20 mM MOPS/1 mM EGTA (pH 7.4 with NaOH) (10 ml/100-mm plate). Cells were scraped from the dishes with a rubber policeman and homogenized in a Brinkmann Polytron (setting 6 for 10 sec). The homogenate was centrifuged at 20,000 × *g* for 20 min and the pellet resuspended in 50 mM MOPS/1 mM EGTA/0.1% BSA (pH 7.5) and used immediately.

Male Wistar rats (200–250 g) were rapidly decapitated, the brains removed, and frontal cortices dissected out and either used immediately or frozen in liquid nitrogen. Chick brains were removed from 20-day embryos and stored in liquid nitrogen. Whole goldfish brains were removed from ice-anesthetized goldfish and used immediately. The tissues were homogenized in a cooled glass homogenizer in 3–5 ml/g tissue of ice cold 20 mM MOPS/1 mM EGTA (pH 7.4). The homogenate was centrifuged at 1000 × *g* for 10 min at 4°C. The supernatant was removed and saved, and the pellet was rehomogenized and centrifuged again. The supernatants were combined and centrifuged at 48,000 × *g* for 20 min. The pellet was resuspended in 2 parts buffer/1 part water and recentrifuged for 20 min at 48,000 × *g*. To solubilize the membranes the pellet was resuspended in 3 ml assay buffer containing 1% Triton X-100 and left on ice for 30 min with occasional vortexing. The solution was centrifuged at 48,000 × *g* for 30 min at 4°C and the supernatant, containing the solubilized membrane proteins, removed and diluted, as appropriate.

Binding Assays

[^{3}H]Phencyclidine binding to *Torpedo* membranes. A centrifugation assay (25) or a modification (21) of the vacuum filtration assay of Eldefrawi *et al.* (26) was used to measure binding of [^{3}H]PCP to nAChR-rich *Torpedo* membrane fragments. The results from the two methods were identical. Membranes (100 nM, expressed in αBgt binding sites) were incubated with 100 nM [^{3}H]PCP for 45 min at 20°C in a total volume of 200 μl of MOPS/EGTA/BSA. This concentration of radioligand was sufficiently low to ensure that binding only to the high affinity site was measured. Nonspecific binding was determined in the presence of 100 μM unlabeled PCP. Reactions were terminated by rapid filtration through glass fiber filters (#32, Schleicher and Schuell, Keene, NH) followed by a single rapid rinse of the filters with incubation buffer. The amount of radioactivity retained on the filters was determined by scintillation counting. For the centrifugation assay, the tubes were centrifuged in an Eppendorf minifuge for 15 min. After removal of an aliquot for measurement of the free concentration of [^{3}H]PCP, the supernatant was aspirated, and the tubes were wiped dry. The pellet was resuspended in 50 μl of 10% Triton X-100, quantitatively removed with two washes of the tip of the centrifuge tube, and scintillation counted to determine membrane-associated radioactivity. The effect of substance P on the equilibrium dissociation constant for [^{3}H]PCP binding to the high affinity binding site was assessed with saturation isotherms. Increasing concentrations of unlabeled PCP (50 nM to 1 μM) were included in the incubation, and the specific activity of PCP was calculated at each free concentration.

[^{3}H]Acetylcholine binding to *Torpedo* membranes. A centrifugation assay as described by Heidmann *et al.* (27) was used to measure [^{3}H]ACh binding to membrane fragments. Membranes (10–20 μM in αBgt binding sites) were incubated with diisopropylfluorophosphate (1.5 mM) for 10 min to prevent

hydrolysis of [^{3}H]ACh by residual acetylcholinesterase. After dilution to 350 nM, the membranes were incubated with 25 nM [^{3}H]ACh for 15 min at 20°C in a total volume of 150 μl of MOPS/EGTA/BSA with 75 μM DFP in 1.5-ml Eppendorf minifuge tubes. After incubation the tubes were centrifuged and the supernatant and pellets counted as described above for [^{3}H]PCP binding. Nonspecific binding was determined in the presence of 5 mM carbamylcholine.

[^{125}I]α-Bungarotoxin binding to *Torpedo*, BC$_3$H-1, and solubilized brain membranes. The initial rate of [^{125}I]αBgt binding was used to determine the effect of substance P on αBgt binding, since equilibrium measurements with a quasi-irreversible ligand are difficult to interpret. Binding of [^{125}I]αBgt was linear for over 2 min. Membranes were preincubated with carbamylcholine or peptide for 15 min at 20°C in MOPS/EGTA/BSA buffer before addition of [^{125}I]αBgt. The concentration of [^{125}I]αBgt was 2 nM for the solubilized brain membranes and 15 nM for all other tissue preparations. The final volumes and membrane concentrations follow: 150 μl, *Torpedo* membranes (4.5 nM expressed in αBgt binding sites) and BC$_3$H-1 membranes (0.4 mg protein/ml); 500 μl, solubilized brain membranes (40 mg tissue wet wt/ml). Initial rates were determined after 20 sec (*Torpedo* and BC$_3$H-1) or 1 min (brain) incubations. For membrane preparations, reactions were terminated by addition of 3 ml buffer and rapid filtration through Millipore GVWP (0.2 μm, *Torpedo*) or HVLP (0.5 μm, BC$_3$H-1) filters presoaked in 1% BSA, followed by three washes with buffer (28). Since it was sometimes found that substance P displaced [^{125}I]αBgt from the filters at high concentrations (>10 μM), the first wash contained substance P, such that all filters were exposed to 5 μM substance P during the first filtration. For soluble *Torpedo* membranes, the solubilized membranes (in 1.0% Triton X-100) in the supernatant of the 100,000 × *g* centrifugation were diluted to 4.5 nM and 0.1% Triton X-100 in MOPS/EGTA/BSA, and the assay was performed as for *Torpedo* membranes, except Whatman DE81 filters were used (24). Binding reactions with solubilized membranes were terminated by addition of 4 ml of 33% saturated ammonium sulfate and filtration of the precipitated protein on Millipore EHWP filters, followed by three washes with 33% ammonium sulfate (29). There was no effect of substance P on nonspecific binding of [^{125}I]αBgt to the filters with this method and the peptide was not included in the wash. Radioactivity retained by the filters was determined in a gamma counter. Nonspecific binding was determined in the presence of 5 mM carbamylcholine.

[^{3}H](−)Nicotine binding to solubilized brain membranes. A vacuum filtration assay was used to measure [^{3}H](−)nicotine binding (30) to various solubilized brain membranes. Solubilized brain membranes (20 mg wet wt/ml) were incubated with 5 nM [^{3}H](−)nicotine in 500 μl of 50 mM MOPS/1 mM EGTA on ice in the dark for 1 hr. The reaction was terminated by rapid filtration on Whatman GF/C or Schleicher and Schuell #30 glass fiber filters presoaked in 0.3% polyethylenimine (31). The filters were washed three times with ice-cold buffer and scintillation counted. Nonspecific binding was determined by including 0.1 mM carbamylcholine in the incubation.

There was no evidence in any filtration assay of "specific" binding to the filters. In the absence of tissue, filter-associated radioactivity was the same for incubation conditions measuring total and nonspecific binding.

Analysis and Statistics

The K_D and B_{max} values for the high affinity binding of [^{3}H]PCP were determined from the saturation isotherms by fitting a single binding site to the data (32) using the nonlinear least-squares method of Marquardt and Levenberg adapted from Bevington (33). The IC_{50} and the apparent n_H were determined from the concentration-inhibition curves by linear regression of the indirect Hill plots, using only the data between 10 and 90% inhibition (32). The IC_{50} values were corrected to K_I values by the method of Linden (34), which corrects for changes in the free concentrations of ligands when the concentration of the binding sites approaches the K_D and K_I. Since [^{125}I]αBgt binding was measured as an initial rate, the IC_{50} was assumed to equal the K_I. Statistical significance for the effects of substance P on [^{3}H]PCP was evaluated by Student's *t* test or *Neuman-Keuls* test as described in the figure and table legends.

Materials

Live *T. californica* were obtained from Pacific Biomarine (Venice, CA) and frozen *T. nobiliana* electroplaque from Biofish Associates (Georgetown, MA). Tissue culture supplies were purchased from Grand Island Biological (Grand Island, NY). Substance P was obtained from Bachem (Torrance, CA). [^{3}H]ACh (86 Ci/mmol, used within 1 week of delivery), [^{3}H]PCP (48 Ci/mmol), [^{3}H](−)nicotine (60 Ci/mmol), and [^{125}I]αBgt (70–140 Ci/mmol) were purchased from New England Nuclear (Boston, MA). All other chemicals were purchased from Sigma Chemical (St. Louis, MO).

Results

Effects of Substance P on [^{3}H]PCP Binding to *Torpedo* Membranes

Because of the similarities in the noncompetitive inhibition of nAChR activation by substance P and local anesthetics, the effect of substance P on the high affinity binding of [^{3}H]PCP was examined. PCP binds with high affinity to a site on the nAChR that mediates the noncompetitive inhibition of receptor activation by local anesthetics (26, 35). In the absence of carbamylcholine, substance P caused a concentration-dependent increase in [^{3}H]PCP binding that was maximal at approximately 30 μM (Fig. 1A). Above this concentration binding decreased but did not return to the control value at the highest concentration examined (316 μM). The peptide-induced increase in binding reflected a decrease in the K_D for [^{3}H]PCP binding with no significant effect on the apparent number of binding sites (B_{max}) (Fig. 2, Table 1). The peptide thus appears to modulate PCP binding allosterically by inducing an increase in affinity of the nicotinic receptor for PCP.

For comparison, the effects on [^{3}H]PCP binding of agents that inhibit nAChR activation via the PCP binding site are also shown in Fig. 1A. As reported by others (35), PCP itself and the local anesthetics dibucaine and dimethisoquin decreased [^{3}H]PCP binding in a manner consistent with a competitive interaction. The K_I values were: PCP 860 nM (n_H = 0.86), dibucaine 3.0 μM (n_H = 0.99), and dimethisoquin 5.0 μM (n_H = 0.99).

In the presence of carbamylcholine, the effect of substance P on [^{3}H]PCP binding was markedly different. Carbamylcho-

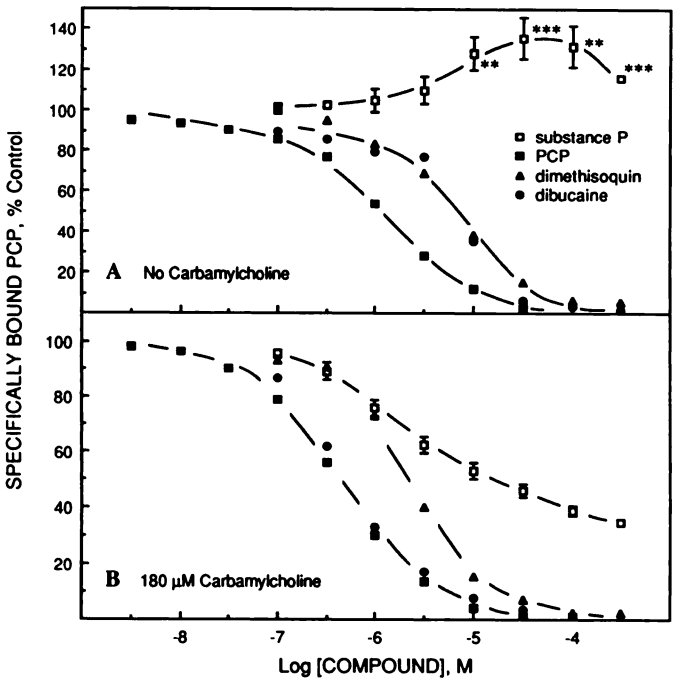

Fig. 1. Effects of substance P, PCP, and local anesthetics on the high affinity binding of [^{3}H]PCP to *Torpedo* acetylcholine receptors. *Torpedo* membranes (100 nM in αBgt sites) were incubated with 100 nM [^{3}H]PCP for 45 min in the presence of increasing concentrations of substance P (□), PCP (■), dibucaine (●), and dimethisoquin (▲) in the absence (A) and presence of 180 μM carbamylcholine (B). Bound [^{3}H]PCP was separated from free by centrifugation or rapid filtration through glass fiber filters. Results were identical using either method of separation. Nonspecific binding, measured in the presence of 100 μM PCP, has been subtracted. In the presence of carbamylcholine, control binding was 251 ± 18% of control binding in the absence of agonist (see Fig. 2). Data points for substance P are means ± SE from six experiments, each performed in duplicate and normalized to control binding in the absence of peptide. PCP and local anesthetic data points are from a representative experiment performed in duplicate. ***Significantly different from control, $p < 0.005$, *t* test; **significantly different from control, $p < 0.025$, *t* test.

line allosterically modulates [^{3}H]PCP binding, presumably by promoting formation of the high affinity desensitized state of the receptor (35). Carbamylcholine (180 μM) increased 100 nM [^{3}H]PCP binding 2.5-fold (Fig. 1). This reflected a 3.2-fold decrease in the K_D with no significant change in B_{max} (Fig. 2, Table 1). Substance P decreased [^{3}H]PCP binding in the presence of carbamylcholine in a concentration-dependent manner but with an extremely low slope (Fig. 1B). The decrease appeared to plateau with a maximal inhibition of approximately 65%. This inhibition of binding reflected an increase in the K_D for [^{3}H]PCP with no significant effect on the B_{max} (Fig. 2, Table 1). The interaction between substance P and [^{3}H]PCP in the presence of carbamylcholine appears to be complex and is more consistent with an allosteric modulation of PCP binding by substance P than a competitive interaction. Also shown in Fig. 1B are the concentration dependences for PCP, dibucaine, and dimethisoquin inhibition of [^{3}H]PCP binding in the presence of carbamylcholine. As seen in the absence of carbamylcholine, these agents decrease binding in a manner consistent with competitive interactions. The K_I values were: PCP 212 nM (n_H = 0.97), dibucaine 240 nM (n_H = 0.99), and dimethisoquin 1.1 μM (n_H = 1.04). As observed for [^{3}H]PCP, carbamylcholine increased the affinity of the local anesthetics between 3- and 8-fold.

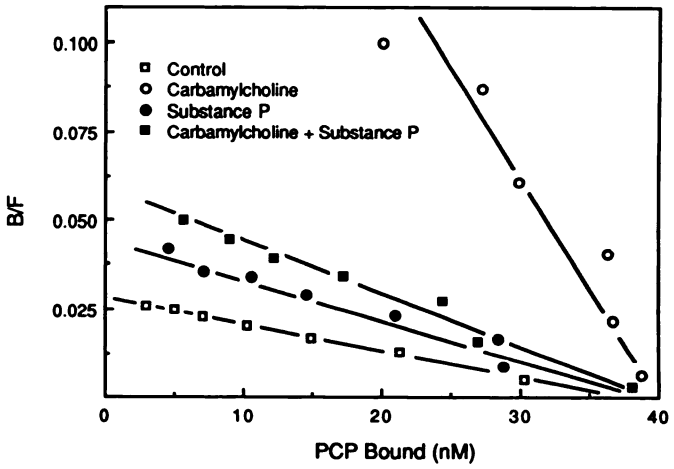

Fig. 2. Scatchard plots of the effect of 30 μM substance P on saturation isotherms of high affinity [^{3}H]PCP binding to *Torpedo* acetylcholine receptors in the absence and presence of carbamylcholine. *Torpedo* membranes (100 nM in αBgt sites) were incubated with increasing concentrations of [^{3}H]PCP (150 nM to 1 μM): no additions (□), 180 μM carbamylcholine (○), 30 μM substance P (●), and 180 μM carbamylcholine + 30 μM substance P (■). Bound [^{3}H]PCP was separated from free by centrifugation. Nonspecific binding, measured in the presence of 100 μM PCP, has been subtracted. Data shown are from a representative experiment performed in duplicate.

TABLE 1

Effects of substance P and carbamylcholine on the parameters for equilibrium binding of [^{3}H]PCP to Torpedo membranes

Equilibrium saturation isotherms for [^{3}H]PCP binding were analyzed as described in Experimental Procedures. K_D and B_{max} values were determined in the presence and absence of 30 μM substance P and 180 μM carbamylcholine. Values are means ± SE. Statistical significance for the differences in the means was determined using paired *t* tests on the B_{max} values and Newman-Keuls test on the logarithms of the K_D values (n = 6).

Condition	[Carbamylcholine]	[Substance P]	K_D[a]	B_{max}[b]
	μM	μM	μM	nM
1	0	0	1.04 ± 0.18	42.6 ± 2.6
2	180	0	0.16 ± 0.01	40.4 ± 1.4
3	0	30	0.69 ± 0.05	35.4 ± 2.3
4	180	30	0.61 ± 0.04	37.2 ± 2.5

[a] All K_D values are significantly different from each other ($p < 0.05$) except condition 3 versus 4.
[b] B_{max} values are not significantly different ($p > 0.10$).

Therefore substance P does not appear to interact directly with the local anesthetic binding site but allosterically modulates its affinity for PCP. This modulation is similar to that observed for nicotinic agonists like carbamylcholine but would appear to have a lower efficacy for affecting PCP affinity. Substance P increases the affinity less than carbamylcholine and partially reverses the increase induced by the agonist.

Effects of Substance P on [^{3}H]ACh Binding to *Torpedo* Membranes

Since substance P appeared to be acting like a partial agonist, the effect of substance P on [^{3}H]ACh binding to *Torpedo* membranes was examined to determine whether it binds to the ACh binding site. Substance P had no significant effect on [^{3}H]ACh binding at equilibrium (Fig. 3). At the higher concentrations of substance P there was a trend toward increased binding; however, there was substantial scatter in the data at these concentrations, and the increase was not statistically significant. The equilibrium dissociation constant for [^{3}H]ACh was 44.3 ± 6.2 nM (n = 3, data not shown). Also shown in Fig. 3 is

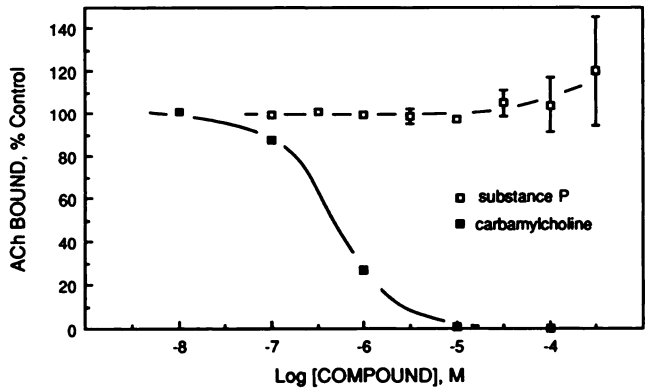

Fig. 3. Effect of substance P on [^{3}H]ACh binding to *Torpedo* acetylcholine receptors. *Torpedo* membranes (350 nM in αBgt sites) were incubated with 25 nM [^{3}H]ACh for 15 min in the presence of increasing concentrations of substance P (□). Bound [^{3}H]ACh was separated from free by centrifugation. Nonspecific binding, measured in the presence of 5 mM carbamylcholine, has been subtracted. Data points are mean ± SE from eight experiments performed in duplicate and normalized to control binding in the absence of peptide. A representative displacement of [^{3}H]ACh by carbamylcholine is also shown (■).

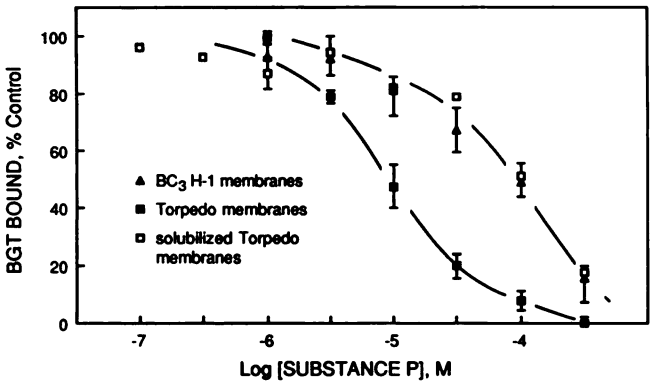

Fig. 4. Effect of substance P on the initial rate of [^{125}I]αBgt binding to acetylcholine receptors from *Torpedo* and BC_3H-1 cells. Native (■) and detergent-solubilized (□) *Torpedo* membranes (4.5 nM in αBgt sites) and BC_3H-1 membranes (▲) (0.4 mg protein/ml) were incubated with 15 nM [^{125}I]αBgt for 20 sec in the presence of increasing concentrations of substance P. Bound [^{125}I]αBgt was separated from free by rapid filtration through glass fiber filters. Nonspecific binding, determined in the presence of 5 mM carbamylcholine, has been subtracted. Data points are means ± SE from three (BC_3H-1 membranes), four (solubilized *Torpedo* membranes), or five (native *Torpedo* membranes) experiments performed in duplicate. Results from each experiment were normalized to control binding in the absence of peptide.

the displacement of [^{3}H]ACh by carbamylcholine (K_I = 280 nM, n_H = 1.31), consistent with an interaction at the agonist binding site. Substance P appears not to bind to the agonist sites on the nAChR, and there is no evidence for allosteric modulation of ACh binding.

Effects of Substance P on [^{125}I]αBgt Binding

Native *Torpedo* membranes. Although substance P had no effect on [^{3}H]ACh binding to *Torpedo* membranes, the peptide was a fairly potent inhibitor of [^{125}I]αBgt binding (Fig. 4). The peptide decreased the initial rate of [^{125}I]αBgt binding with a K_I value of 11.54 ± 2.95 μM (n_H = 1.02 ± 0.07; n = 5). Consistent with previous reports and the values reported above for inhibition of [^{3}H]ACh binding, carbamylcholine inhibited the initial rate of [^{125}I]αBgt binding with a K_I value of 220 nM (n_H = 1.45). This inhibition of αBgt binding by substance P is consistent with a competitive interaction, although this could reflect either binding directly to the αBgt site or an allosteric modulation of αBgt binding. From this inhibition of [^{125}I]αBgt binding and the lack of effect on [^{3}H]ACh binding, substance P seems to differentiate the αBgt binding site from the ACh binding site on the nAChR in *Torpedo* membranes, suggesting that they are not identical sites or that they only partially overlap.

Solubilized *Torpedo* membranes. Although 20–50% of the protein in the nAChR-enriched *Torpedo* membranes is nAChR-associated, it could not be eliminated that a separate protein may be mediating the effects of substance P on receptor binding. To determine whether substance P was interacting directly with the nAChR, membranes were solubilized in 1.0% Triton X-100 and the effects of the peptide on [^{125}I]αBgt binding examined. We reasoned that the high detergent-to-protein ratio should separate all proteins in the membranes such that each micelle would contain at most one protein. Only if substance P were binding directly to the nAChR or to a tightly associated protein would substance P have an effect on [^{125}I]αBgt binding after membrane solubilization. As shown in Fig. 4, substance P inhibited [^{125}I]αBgt binding even after membrane solubilization. There was, however, a significant increase in the K_I value (92.5 ± 5.8 μM; n_H = 1.17 ± 0.10; n = 3). These data support the hypothesis that substance P interacts directly with the nAChR, although solubilization may decrease the apparent affinity of the interaction.

BC_3H-1 membranes. Substance P also decreased the initial rate of [^{125}I]αBgt binding to BC_3H-1 membranes in a concentration-dependent manner (Fig. 4). However, the K_I value (108 ± 23 μM; n_H = 1.00 ± 0.06; n = 3) was significantly higher than that observed for native *Torpedo* membranes. This difference may at least partially reflect the large difference in the protein concentrations in the assays (400 versus 1 μg/ml).

Brain membranes. To examine whether substance P interacts with other nAChRs, suggesting a general modulatory role for the peptide, the effect of the peptide on [^{125}I]αBgt binding to solubilized brain membranes from many species was investigated. Although the relationship between [^{125}I]αBgt binding sites and nAChRs in neuronal tissues is still controversial, there is accumulating evidence that [^{125}I]αBgt may bind to a subpopulation of nAChRs (36–40). Substance P inhibited the initial rate of [^{125}I]αBgt binding to solubilized membranes from rat cortex, goldfish brain, and chick brain in a concentration-dependent manner, although in all tissue preparations inhibition was incomplete (Fig. 5). The peptide was most potent in rat cortex, decreasing the initial rate by 50% at 3 μM. Also shown in Fig. 5 are the concentration dependences for inhibition of [^{125}I]αBgt binding by (−)nicotine and carbamylcholine. These nicotinic agonists completely inhibited specific binding in the micromolar range, as expected for interactions with a nAChR (K_I values: carbamylcholine 2–10 μM, (−)nicotine 0.3–1.5 μM). The apparent Hill coefficients for these agonists are significantly less than 1.0 (carbamylcholine 0.3–0.8, (−)nicotine 0.4–0.8), consistent with multiple agonist binding affinities for the αBgt binding sites in neuronal tissues, as has been reported (36). Thus, in many brain membrane preparations substance P appears to interact with a putative subpopulation of neuronal nAChRs that are labeled by [^{125}I]αBgt. The peptide decreases the initial rate of αBgt binding to these sites in the same

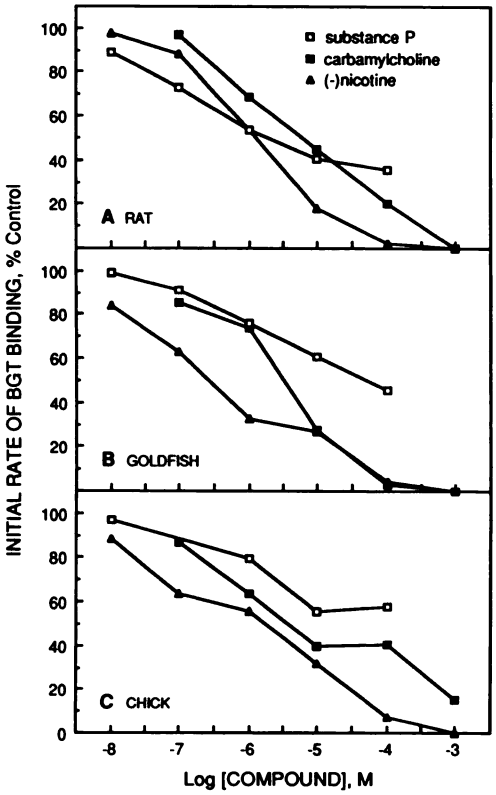

Fig. 5. Effect of substance P and cholinergic agents on the initial rate of [^{125}I]αBgt binding to solubilized brain membranes. Solubilized rat cortex (A), goldfish brain (B), and chick brain (C) membranes (40 mg wet wt/ml) were incubated with 2 nM [^{125}I]αBgt for 1 min in the presence of increasing concentrations of (−)nicotine (▲), carbamylcholine (■), and substance P (□). Bound [^{125}I]αBgt was separated from free by rapid filtration through Millipore EHWP filters after addition of 33% ammonium sulfate. Nonspecific binding, determined in the presence of 5 mM carbamylcholine, has been subtracted. Data points are from a representative experiment done in duplicate.

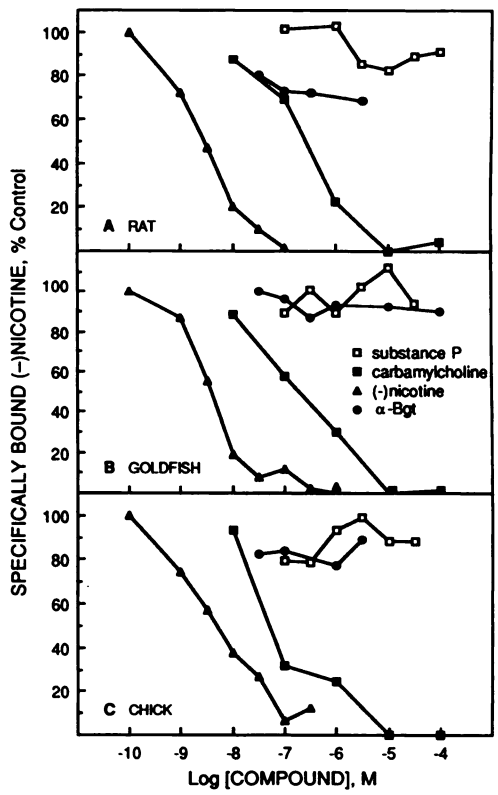

Fig. 6. Effect of substance P and cholinergic agents on the equilibrium binding of [^{3}H](−)nicotine to solubilized brain membranes. Solubilized rat cortex (A), goldfish brain (B), and chick brain (C) membranes (20 mg wt/ml) were incubated with 5 nM [^{3}H](−)nicotine for 1 hr in the presence of increasing concentrations of (−)nicotine (▲), carbamylcholine (■), αBgt (●), and substance P (□). Bound [^{3}H](−)nicotine was separated from free by rapid filtration through polyethylenimine-treated glass fiber filters. Nonspecific binding, determined in the presence of 0.1 mM carbamylcholine, has been subtracted. Data points are from a representative experiment performed in duplicate.

concentration range as for muscle-type nAChRs in *Torpedo* electroplaque and BC_3H-1 cell membranes.

Effects of Substance P on [^{3}H](−)Nicotine Binding to Brain Membranes

The effect of substance P on [^{3}H](−)nicotine binding to the solubilized brain membrane preparations was also examined. [^{3}H](−)nicotine appears to be a specific high affinity ligand for putative brain nAChRs (30). As discussed above, the relationship between the (−)nicotine and αBgt sites in brain is controversial. We observed no effect of substance P and only a slight effect of αBgt on the equilibrium binding of [^{3}H](−)nicotine in these brain preparations (Fig. 6), although (−)nicotine and carbamylcholine were potent inhibitors of binding (K_I values: (−)nicotine 8–12 nM, carbamylcholine 50–100 nM).

Discussion

The noncompetitive inhibition of nAChR activation by substance P is similar in many ways to that produced by local anesthetics. Both substance P and local anesthetics appear to enhance desensitization (10, 15, 16) and to shorten the open channel lifetime of the nAChR (17). However, the results of the present study suggest that substance P does not interact directly with the high affinity binding site labeled by [^{3}H]PCP. This site appears to mediate the noncompetitive inhibition of nAChR activation by local anesthetics (27). In the absence of agonist, substance P allosterically increases the affinity of this site for [^{3}H]PCP. In contrast, the local anesthetics dibucaine and dimethisoquin displaced [^{3}H]PCP binding in a manner consistent with a competitive interaction, as has been reported (27, 35). In the presence of carbamylcholine, an allosteric modulator of PCP binding, substance P decreased the affinity of [^{3}H]PCP binding, and the inhibition was complex and inconsistent with a competitive interaction. This inhibition appears also to reflect an allosteric modulation by substance P of [^{3}H]PCP binding in the presence of agonist. We interpret these results as strong evidence that substance P is not inhibiting nAChR activation by interacting with the high affinity local anesthetic binding site, although the peptide allosterically induces effects at this site.

Substance P does not appear to be acting through a high affinity agonist binding site, since the peptide did not inhibit [^{3}H]ACh binding. In contrast, there was a trend toward the peptide increasing [^{3}H]ACh binding, although the changes were not statistically significant. If substance P were modulating nAChR function by allosterically stabilizing the desensitized state, the peptide would be expected to increase the apparent affinity of an agonist, as has been observed for local anesthetics (16). Our results would be consistent with the peptide affecting

only the kinetics of desensitization without altering the equilibrium constants of isomerization. However, we have recently shown that substance P affects both the rate and extent of desensitization in PC12 cells (15), which would indicate an allosteric modulation of the equilibrium constants. The reason for this discrepancy is unclear, although it may reflect differences between the nAChRs in PC12 cells (neuronal type) and *Torpedo* electroplaque (muscle type). These data do not eliminate an interaction of substance P with the putative low affinity agonist binding site(s) on the nAChR (41, 42). Low affinity binding sites for acetylcholine may mediate activation (K_D = 80 μM (40)) and inhibition (K_D = 800 μM (41)) of the nAChR. The concentrations of [^{3}H]ACh used in our binding studies would not detect these sites, so it is possible that the peptide exerts its effect through a low affinity acetylcholine binding site.

Substance P inhibited the initial rate of [^{125}I]αBgt binding to muscle-type nAChRs in both *Torpedo* and BC_3H-1 membranes in a concentration-dependent manner. The inhibition was consistent with a competitive interaction between substance P and αBgt, which could reflect either competition for the same binding site or an allosteric interaction. Substance P may thus interact with nAChRs in general, as we proposed in studies of the functional effects of the peptide (11). Substance P inhibition of [^{125}I]αBgt binding to *Torpedo* nAChRs with no effect on [^{3}H]ACh binding could mean that αBgt and ACh bind to different sites on the receptor; however, it is just as likely that substance P and ACh interact with different subsites within the αBgt binding site.

After detergent solubilization of *Torpedo* membranes, substance P still inhibited [^{125}I]αBgt binding, although the K_I increased 10-fold; 3- to 10-fold decreases in apparent affinity after detergent solubilization have been reported for local anesthetic binding to the high affinity PCP binding site (22). These decreases probably reflect conformational changes in the nAChR when the detergent micelle environment is substituted for the native lipid environment. Since the protein-to-detergent ratio was extremely low during solubilization, it is very likely that there is only one protein per micelle. Inhibition of [^{125}I]αBgt binding by substance P after detergent solubilization suggests that the peptide is binding to the nAChR itself or a tightly associated protein that does not dissociate during solubilization. To answer this question more directly, we are currently purifying the solubilized nAChR and reconstituting it into liposomes. Demonstration of an effect of substance P on [^{125}I]αBgt and [^{3}H]PCP binding in this system would be compelling evidence for a direct interaction of substance P with the nAChR.

Substance P also inhibited [^{125}I]αBgt binding to the various brain membranes examined. Although inhibition in these tissues was incomplete, these data suggest a general modulatory action for substance P on nAChRs. The relationship between [^{125}I]αBgt binding sites and nAChRs in neuronal tissues is controversial, although it has been suggested that [^{125}I]αBgt may bind to a subpopulation of nAChRs in neuronal tissues (37–40). There is also recent evidence that there are at least two different binding sites for agonists within the subpopulation of receptors labeled by αBgt in brain membranes (36). The presence of multiple binding sites with different affinities causes n_H values for agonist inhibition of αBgt binding to be significantly less than 1.0 (Fig. 5; see also Ref. 36). The incomplete inhibition of αBgt binding by substance P could reflect an interaction of the peptide with a subset of these sites. Substance P had no effect on [^{3}H](−)nicotine binding to these brain membranes, which is consistent with the lack of effect on [^{3}H]ACh binding to *Torpedo* nAChRs. These results may thus reflect different actions of substance P on the various putative subpopulations of neuronal nAChRs labeled by [^{125}I]αBgt and [^{3}H](−)nicotine (37–40).

In summary, the results of this study suggest that substance P does not inhibit nAChR activation by binding to the high affinity local anesthetic binding site, but the peptide allosterically modulates binding to this site in a manner similar to agonists. The peptide also does not appear to interact directly or indirectly with the high affinity agonist binding site. Substance P may bind to a site on the receptor that at least partially overlaps the α-neurotoxin binding site, since [^{125}I]αBgt binding to *Torpedo* nAChR can be completely inhibited by the peptide. In addition, substance P may interact with a subpopulation of nAChRs in the central nervous system, indicating a possible physiological role for the peptide in the modulation of nAChR function in the brain.

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