DIRECT BINDING STUDIES OF ^{125}I-α-BUNGAROTOXIN AND ^{3}H-QUINUCLIDINYL BENZILATE INTERACTION WITH AXON PLASMA MEMBRANE FRAGMENTS. EVIDENCE FOR NICOTINIC AND MUSCARINIC BINDING SITES

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ABSTRACT. The attachment of ^{125}I-α-bungarotoxin (BgTx) which is reportedly bound exclusively to "nicotinic" acetylcholine receptors, as well as ^{3}H-atropine and ^{3}H-3-quinuclidinyl benzilate (QNB), which reportedly bind exclusively to "muscarinic" receptors, was measured in isolated lobster axon plasma membrane fragments and in the soluble axonal protein fraction. ^{125}I-α-BgTx binding was also measured in lysolecithin-solubilized fragments. Binding assays were adapted for these studies and are described in detail. High affinity, saturable binding of all three ligands to membrane fragments was observed, as well as binding of BgTx to a macromolecule present in both the soluble fraction and the membrane fragments. These experiments provide the first evidence for the very tight binding of both "nicotinic" and "muscarinic" ligands in peripheral nerve.

For many years a major controversy has been centered on the postulate by Nachmansohn (1) that acetylcholine (ACh) plays a central role in both axonal and synaptic conduction. While a great deal of information is available about ACh receptors in postsynaptic membranes, only recently have studies been initiated to investigate the presence of such receptors in axonal membranes (2-5). Initial findings are compatible with earlier evidence that cholinergic ligands can be attached to axonal membranes once permeability barriers are removed (6,7).

The present study demonstrates, by direct binding experiments, the tight attachment of ^{125}I-α-bungarotoxin (BgTx) and of ^{3}H-quinuclidinyl benzilate (QNB) to axon plasma membrane fragments. The former ligand has been reported to bind to ACh receptors in tissues affected by nicotinic agonists or antagonists (8), and the latter ligand is said to bind specifically to receptors affected by muscarinic agents (9). Direct binding techniques were also used to determine binding constants for ^{14}C-ACh and ^{3}H-atropine. These experiments strongly

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suggest the presence of a cholinergic system in nerve membranes which may be similar to that observed in other electrically excitable membranes.

MATERIALS AND METHODS. The preparation of axon plasma membrane fragments from lobster (Homarus americanus) walking leg nerve bundles was described in detail by Denburg (10). Lobsters were purchased in Boston from the James Hook Co. [^{3}H]-Quinuclidinyl benzilate (specific activity 8.4 Ci/mmol) and [^{3}H]-atropine (specific activity 1.2 Ci/mmol) were purchased from Amersham/Searle [^{125}I]-α-Bungarotoxin was a gift from Dr. Hai-Won Chang.

LIGAND-BINDING MEASUREMENTS. ULTRAFILTRATION METHOD. The binding of [^{3}H]-QNB and [^{3}H]-atropine to axon plasma membrane protein was assayed by the filtration method developed by Paulus (11) using an 8-channel ultrafiltration cell (M-129) manufactured by MRA Corp., Boston, Mass. The protein (about 50 µg) and radiolabeled ligand were incubated for 1-4 hrs. at 5°C. 100 µl Aliquots of the solutions were then subjected to filtration under 40 p.s.i. at room temperature through Amicon UM-10 Diaflo filter discs (1 cm. diam.), exhaustively washed with water shortly before use to remove the glycerol preservative. These membranes will retain macromolecules with molecular weights in excess of 10,000. After filtration, the membranes were washed under pressure with 1 volume of buffer, and the underside of the filters was washed with 5 ml of ethylene glycol injected through rinse channels in the lower block of the apparatus. The membranes were then transferred to scintillation vials containing 1 ml of dimethylformamide to solubilize the outer layer of the filter material and insure maximum extraction of counts. 15ml of Beckman GP scintillation fluid were added for counting.

Blank values were determined by filtering samples of the radioactive ligand without protein to correct for the small volume of solution (3-5 µl) retained by the Diaflo membranes. To further reduce the error from variations in the nonspecific binding of ligand to the filter discs, a blank was run in parallel with every test concentration of ligand plus protein. The UM-10 Diaflo membranes were chosen especially for their low levels of nonspecific binding of ^{3}H-QNB (5-10%) and ^{3}H-atropine (1-5%). Concentration-dependence, volume-dependence and saturation of the level of nonspecific binding of both ligands was noted.

GEL FILTRATION METHOD. The binding of mono-iodinated ^{125}I-α-BgTx was assayed by ion-exchange chromatography on 1ml CM-50 (Pharmacia) columns equilibrated in buffer (10mM Tris, pH 7.8) at room temperature. Free toxin is bound to the carboxymethyl sephadex and the membrane protein:toxin complex is eluted in the void volume (about 1.2 ml) of the column. The samples were prepared by incubating 150-200 µg of crude membrane protein with radiolabelled toxin in 200 µl of buffer for 60-90 mins. at room temp. The solutions were then applied to the CM-50 columns and eluted with 1.2 ml of buffer in 3 400 µl fractions. 100 µl Aliquots of the column fractions were counted in scintillation vials containing 5 ml of Beckman BP counting fluid, and the protein concentrations of the fractions were determined by the Lowry assay (12).

SOLUBILIZATION WITH LYSOLECITHIN. The nicotine-binding component of axon plasma membrane fragments is inactivated by sonication and by several detergents, including Triton X-100 and Tween 80, but it is solubilized in high yield (90% of total protein) with a 3:1 weight ratio of lysolecithin (lysophosphatidyl choline, Sigma), as previously demonstrated by Denburg (13). The solubilized fraction retains full nicotine-binding activity and was assayed, along with a Triton X-100 solubilized fraction, for α-BgTx binding activity, after dialysis for 24 hrs. to remove excess detergent.

TABLE I. Dissociation constants (K_D) and maximum binding capacity (B_{Max}) for ^{125}I-α-bungarotoxin in axon plasma membrane and soluble axonal proteins from lobster walking leg nerve bundles.*

AXONAL PROTEIN	K_D (M x 10^{-7})	B_{Max} (pmoles/mg protein)
Pellet (100,000 x g)	2.7 ± 1.0	83 ± 7
Supernatant (100,000 x g)	2.4 ± 1.0	17 ± 4
Solubilized pellet (lysolecithin)	1.4 ± 0.9	44 ± 4

*The data are presented as the mean ± the standard error of 6 to 10 experiments (total # of points) on a single axonal fraction and were determined by extrapolation of double-reciprocal plots with least squares regression analysis.

Dissociation constants for the various ligands were determined by extrapolation of double-reciprocal plots of the ligand-binding data with the use of computed least squares regression lines.

RESULTS AND DISCUSSION

α-BUNGAROTOXIN BINDING. Table I shows the dissociation constants and maximum high-affinity binding capacity for ^{125}I-α-BgTx measured in axonal membrane fragments (pellet), soluble axonal proteins (supernatant) and lysolecithin solubilized membrane fragments. The binding constants are statistically equivalent for all 3 protein fractions: pellet = 2.7 ± 1.0 x 10^{-7}M, supernatant = 2.4 ± 1.0 x 10^{-7}M, solubilized pellet = 1.4 ± 0.9 x 10^{-7}M, but the maximum toxin-binding capacity (B_{Max}) is greatest in the pellet. Each of these data points was computed from experiments on a single membrane fraction, as the B_{Max} was seen to vary widely among preparations, probably as a function of contamination by non-binding components. The binding constants, however, remained remarkably uniform. Membrane fragments solubilized with Triton X-100 (1%) or Lubrol WX (1%) were seen to bind very low levels of ^{125}I-α-BgTx when compared

with the binding capacity of membrane fragments or lysolecithin-solubilized membranes. Additional work is in progress to characterize the binding abilities of the solubilized membrane fragments. Controls demonstrated that several non-neuronal membrane and soluble proteins do not bind significant levels of toxin. These include: acetylcholinesterase, bovine serum albumin, ribonuclease, liver microsomal membranes (14), and erythrocyte membrane fragments (15).

Unlike nicotine-binding activity, which is restricted exclusively to the membrane fragments (5,10), α-BgTx-binding activity was found in both membrane-bound and soluble axonal proteins. It was considered possible that toxin-binding protein may be loosely associated with the axonal membrane by an interaction dissociable in high salt. However, preparation of the high-speed (100,000 x g) fraction with 100mM $MgSO_4$ or 0.5-1.0M NaCl failed to elicit any increased "leak" of toxin-binding activity into the soluble fraction.

Using the equilibrium dialysis procedure described previously (5) and acetyl-1-^{14}C-choline iodide (sp. act. 4.53mCi/mmol, New England Nuclear) in the presence of 2 x 10^{-5}M eserine, a K_D of 6.7 x 10^{-6}M for ACh was calculated from a double reciprocal plot of the dose-response data. In earlier studies Denburg et al.(2) determined the affinity of axonal membranes for ACh indirectly, by measuring the inhibition of ^{3}H-nicotine binding, and calculated a K_i of 43 μM.

Balerna et al. (4) confirmed the presence of a nicotine-binding fraction in lobster (Panulirus argus) walking leg nerve membranes but observed no association of Naja nigricollis mossambica neurotoxin with either lobster or crab (Maia squinado) nerve membranes. Denburg et al. (2) measured the binding of α-BgTx to lobster (Homarus americanus) axon plasma membrane indirectly by competiti with ^{3}H-nicotine, using an equilibrium dialysis procedure with cellulose dialysis tubing (16), and found inhibition of nicotine-binding by about a 100-fold excess of toxin. The present measurements of binding constants for the specific cholinergic antagonist α-BgTx as well as for the classical agonist ACh, by

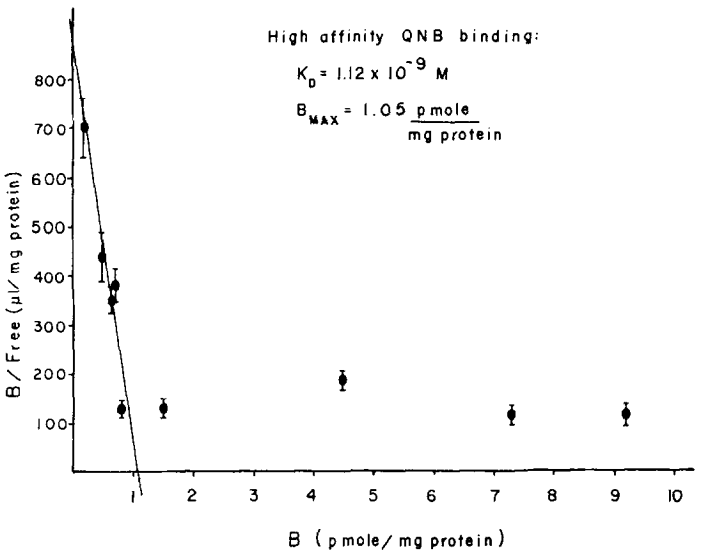

Figure 1. Scatchard analysis of dose-response data for the binding of ^{3}H-QNB to lobster axon plasma membrane fragments. High affinity binding: K_D = 1.12 x 10^{-9}M, maximum = 1.05 pmoles/mg protein. Standard error flags represent the standard error of measurement by the ultrafiltration procedure.

using radiolabelled forms of these ligands, provide by far the most convincing evidence for a cholinergic receptor-like macromolecule in a peripheral nerve membrane.

QNB AND ATROPINE BINDING. The muscarinic antagonist ^{3}H-QNB has been shown to bind with high affinity to acetylcholine receptor protein of mammalian brain, forming an attachment that is not antagonized by nicotinic agents or noncholinergic drugs (9). In the present study, specific, saturable binding of ^{3}H-QNB to axon plasma membrane was detectable at very low concentrations of ligand (less than 10^{-8}M) and is readily masked by non-specific, non-saturable attachment at higher ligand concentrations. This is shown clearly in the Scatchard plot of fig. 1 which yields a high-affinity binding site with a K_D of 1.12 x 10^{-9}M and a B_{Max} of 1.05 pmoles/mg protein and non-saturable binding at QNB concentrations greater than 1 x 10^{-8}M. In addition, a double-reciprocal plot of the dose-response data for 1 x 10^{-8}M to 8 x 10^{-8}M

^{3}H-QNB yields a straight line which intersects the origin of the plot.

Denburg et al. (2) have previously shown that atropine inhibits the binding of ^{3}H-nicotine to axonal membranes, but they did not analyze the binding characteristics of atropine by direct measurements. Utilizing the ultrafiltration technique, we have demonstrated that a specific, saturable component of axon plasma membrane fragments binds the muscarinic antagonist ^{3}H-atropine with a K_D of 6.8 $\pm$ 2.3 x 10^{-7}M and B_{Max} of 59 $\pm$ 15 pmoles/mg protein. It was determined that neither QNB nor atropine, in contrast to BgTx, is attached to soluble proteins in the 100,000 x g supernatant.

Preliminary isoelectric focusing of the soluble and pellet fractions on polyacrylamide gels (17) exhibited marked homogeneity of protein bands in the two fractions (D. C. Hilt and J. K. Marquis, unpublished results), suggesting that α-BgTx can be attached to its receptor macromolecule whether membrane bound or not, while nicotine, QNB and atropine can bind only to receptors in situ.

Preliminary studies of the effects of "muscarinic" agents on the binding of "nicotinic" agents and vice-versa demonstrated that both nicotine and atropine (500 nM) inhibit the binding of 3 nM ^{3}H-QNB by about 50%. The fact that ligand binding is saturable at low concentrations and can be inhibited by receptor agonists or antagonists "known" to be muscarinic or nicotinic is by no means sufficient evidence to ensure that the binding involves one or more pharmacologically relevant receptors. The present data, in fact, suggest a complex relationship of multiple binding sites in axon plasma membrane. To define the nature of the binding sites, the effects on ligand-receptor interactions of a wide range of specific and nonspecific drugs must be evaluated. Such studies are presently in progress.

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