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^{125}I-LABELED α-BUNGAROTOXIN AND [^{3}H]QUINUCLIDINYLBENZILATE BINDING TO RAT BRAIN MEMBRANES

EFFECTS OF PHYSICAL, CHEMICAL AND ENZYMATIC TREATMENTS

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Summary

The effects of various physical, chemical and enzymatic treatments of rat brain membranes were investigated with respect to ^{125}I-labeled α-bungarotoxin ([^{125}I]αBuTx) and [^{3}H]quinuclidinylbenzilate ([^{3}H]QNB) binding. Binding appeared relatively stable to autolysis, mechanical shearing, freeze-thawing, and divalent cation addition (Sr^{2+}) or removal (EGTA, EDTA). Binding for [^{125}I]-αBuTx was slightly reduced by trypsin digestion of the membranes while both [^{125}I]αBuTx and [^{3}H]QNB binding were reduced by phospholipase A_2 digestion (*Crotalus adamantus* phospholipase A_2 and β-bungarotoxin). Treatment of the membranes with the disulfide reducing agent, dithiothreitol, resulted in additional [^{125}I]αBuTx binding but showed little effect on [^{3}H]QNB binding. Binding of the cholinergic agonists, nicotine and carbamylcholine, was studied by observing their concentration-dependent ability to inhibit [^{125}I]αBuTx and [^{3}H]QNB binding, respectively. Membrane sulfhydryl group reduction and endogenous cation removal by EGTA or EDTA resulted in a lowered affinity for nicotine binding. Alkylation of membranes with *N*-ethylmaleimide resulted in an increase in carbamylcholine affinity. Other treatments had little or no effect on nicotine or carbamylcholine binding.

Abbreviations: [^{125}I]αBuTx, ^{125}I-labeled α-bungarotoxin; [^{3}H]QNB, [^{3}H]quinuclidinylbenzilate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

Introduction

Binding of ^{125}I-labeled α-bungarotoxin ([^{125}I]αBuTx) and [^{3}H]quinuclidinyl-benzilate ([^{3}H]QNB) to brain membranes has attracted considerable attention in recent years with their tentative identification as ligands for the recognition sites of nicotinic and muscarinic acetylcholine receptors [1—4]. This identification has been based largely on analogous biochemical studies using peripheral tissue containing nicotinic and muscarinic acetylcholine receptors such as skeletal and smooth neuromuscular junctions [5,6]. In neuromuscular preparations, the identification of ligand binding sites as neurotransmitter receptor-recognition sites is less tentative than in brain, since quantitative pharmacological and physiological studies can be directly correlated with in vitro binding parameters [5—10]. Since quantitiative pharmacological and physiological studies are lacking for most central nervous system receptors, only the biochemical identification of receptor sites and the biochemical quantitation and determination of the properties of these sites are available to guide future physiological studies.

We have recently developed a simple reliable procedure to obtain a synaptic membrane-enriched subcellular fraction from rat brain [11] and report here the further characterization of this membrane fraction with respect to various physical, chemical and enzymatic treatments on [^{125}I]αBuTx and [^{3}H]QNB binding. In addition to the perturbation of putative acetylcholine receptor antagonist binding, we have indirectly examined the effects of various treatments on cholinergic agonist binding by comparing the concentration-dependent ability of nicotine and carbamylcholine to prevent [^{125}I]αBuTx and [^{3}H]-QNB binding. A preliminary account of some of our results has been presented [12].

Materials and Methods

Preparation and treatment of synaptic plasma membrane-enriched fractions. Synaptic plasma membrane-enriched fractions were prepared by adapting the original procedure of Jones and Matus [13] to use a fixed-angle Beckman type 30 rotor [11]. The resulting fraction is enriched 4—6-fold in 'specific' (see below) [^{125}I]αBuTx and [^{3}H]QNB binding compared to the total particulate fraction. Membranes were treated immediately or stored frozen at —20°C until treatment.

A variety of physical, chemical and enzymatic treatments were tested for their effects on [^{125}I]αBuTx and [^{3}H]QNB binding to membranes. The details are presented in the appropriate figure and table legends.

[^{125}I]αBuTx and [^{3}H]QNB binding assay. [^{125}I]αBuTx and [^{3}H]QNB binding were determined by standard assay [15]. Briefly, 0.3 mg of membrane protein was incubated with $3 \cdot 10^{-9}$ M [^{125}I]αBuTx or $4 \cdot 10^{-9}$ M [^{3}H]QNB for 90 min at room temperature in 50 mM Tris, 50 mM NaCl, 0.02% NaN_3 buffer, pH 7.3. Separation of free and bound ligand was accomplished by centrifugation for [^{125}I]αBuTx and filtration for [^{3}H]QNB. After suitable washing of the membranes, bound radioactivity was determined by γ-counting for [^{125}I]-αBuTx and liquid scintillation counting for [^{3}H]QNB. Specific binding for

[^{125}I]αBuTx is defined as the binding blocked by a 1000-fold excess of unlabeled αBuTx, and specific binding for [^{3}H]QNB is that which is blocked by a 1000-fold excess of atropine sulfate. These conditions are adequate to saturate nearly all specific binding sites and result in minimum 'nonspecific' binding.

Agonist binding. Agonist binding was determined indirectly by preincubating aliquots of membrane (0.3 mg) for 30 min at room temperature with various concentrations of nicotine or carbamylcholine in Tris, NaCl, NaN_3 buffer. [^{125}I]αBuTx (for nicotine-pretreated membranes) or [^{3}H]QNB (for carbamylcholine-pretreated membranes) was then added to a final concentration of 1 nM, and the samples were incubated for an additional 90 min. Free and bound ligand were then separated in the usual way. Changes in agonist binding affinity were inferred from a displacement in the nicotine or carbamylcholine inhibition curve relative to untreated membranes.

Materials

[^{125}I]αBuTx (monoiodo, 80—160 Ci/mmol) and [^{3}H]QNB (29.4 Ci/mmol) were obtained from New England Nuclear Corp. Unlabeled α-bungarotoxin was purified according to the method of Mebs et al. [16]. DNAase I, trypsin (TPCK-treated) and *Crotalus adamantus* phospholipase A_2 was obtained from Worthington Biochemical Corp., Freehold, NJ. *Vibro cholerae* neuraminidase and *Bacillus thermoproteolyticus* protease were from Sigma Chemical Co., St. Louis, MO. β-Bungarotoxin was purified from *Bungarus multicinctus* venom [17]. All other chemicals and reagents were of the highest grade commercially available.

Results

Binding of [^{125}I]αBuTx and [^{3}H]QNB to membranes appeared quite stable to a variety of physical, chemical and enzymatic treatments. Table I shows the results for the physical perturbation of the membranes. No change in the amount of [^{125}I]αBuTx binding was observed for membranes prepared at 22°C or subjected to increased mechanical or liquid shear forces. The [^{3}H]QNB binding also remained constant except for a slight increase in specific activity of binding (17%) when the membranes were prepared at 22°C. In all cases, at least 95% of the total protein was recovered after treatment.

Table II shows the results when the membranes were treated with the detergent, digitonin, the chaotropic agent, urea, and low concentrations of two aldehydes, paraformaldehyde and glutaraldehyde. The particulate [^{125}I]αBuTx binding was completely abolished by 6 M urea but appeared relatively stable to the three other treatments. In fact, a 39% increase in specific activity was observed when membranes were treated with 0.1% glutaraldehyde. Apparently, no previously undetected binding was uncovered by this treatment, since 95% of the control binding was recovered after glutaraldehyde treatment. Treatment of membranes with a 10-fold higher concentration of glutaraldehyde or paraformaldehyde produced variable results of either no change or significant reduction of binding (data not shown). In contrast, the [^{3}H]QNB binding appeared less stable after paraformaldehyde treatment and was completely abolished at higher concentrations. Only 77% of the [^{3}H]QNB binding was recovered in the

TABLE I

EFFECT OF VARIOUS PHYSICAL TREATMENTS ON [^{125}I]αBuTx AND [^{3}H]QNB BINDING TO BRAIN MEMBRANES

Values represent means of triplicate determinations repeated at least once. Control values ranged from 20 to $60 \cdot 10^{-15}$ mol/mg for [^{125}I]αBuTx and 0.9 to $2.9 \cdot 10^{-12}$ mol/mg for [^{3}H]QNB. Standard errors were less than 10% for a given set of triplicates and 95% of all protein was recovered after treatment.

Treatment	[^{125}I]αBuTx (% of control)	[^{3}H]QNB (% of control)
Membranes prepared at 22°C *	92.8	117.6
Freeze-thaw (three cycles) **	101.0	106.0
Polytron (30 s top speed) ***	95.6	91.8

* Entire procedure performed as described in Ref. 11 except all operations were performed at 22°C. Total preparation time was 6 h.

** Freshly prepared membranes were rapidly frozen in dry ice/acetone and thawed at room temperature.

*** A 10 mg/ml aliquot of membrane was treated in an ice bath using a Brinkman polytron with a PT-10 generator.

membrane fraction after digitonin treatment, while 57% resisted denaturation and/or solubilization with 6 M urea. The specific activity of [^{3}H]QNB binding increased slightly after treatment with 0.1% glutaraldehyde although only 65% of the protein was recovered after this treatment.

Neurotransmitter receptors often function by being coupled to ion channels, and various ions may influence the binding properties of receptor ligands. Table III presents the results of the addition (Ca^{2+} and Sr^{2+}) or removal of divalent cations on antagonist binding. The added cations and chelating agents were removed prior to binding assay so the results represent stable changes in ligand binding and do not reflect more transient conformational changes. Little effect was observed on [^{3}H]QNB binding by adding exogenous Ca^{2+} or Sr^{2+} or by removing endogenous Ca^{2+} or other divalent cations with EGTA or EDTA. [^{125}I]αBuTx binding was also not affected much by removal of divalent cations. Sr^{2+} seemed to result in a slight increase in [^{125}I]αBuTx binding.

Chemical modification of protein side chains, in particular, disulfide bonds

TABLE II

EFFECT OF DIGITONIN, UREA AND ALDEHYDE TREATMENT ON [^{125}I]αBuTx AND [^{3}H]QNB BINDING TO BRAIN MEMBRANES

See Table I for control values. Greater than 90% of all protein was recovered except for urea (70% recovery) and glutaraldehyde (65% recovery) treatments. Treatment:aliquots of membrane (4—10 mg) were incubated 30 min at room temperature in 6 ml of Tris, HCl, NaN_3 buffer with the indicated concentration of reagent. Membranes were then sedimented at 150 000 × $g \cdot$ min at 4°C and resuspended in a minimum volume of Tris, HCl, NaN_3 buffer in order to remove excess reagent.

Treatment	[^{125}I]αBuTx (% of control)	[^{3}H]QNB (% of control)
Digitonin (0.08%)	111.0	77.0
Urea (6 M)	0.0	57.8
Paraformaldehyde (0.5%) *	94.7	50.8
Glutaraldehyde (0.1%) *	139.0	107.0

* Treated samples were washed twice with 6 ml Tris, NaCl, NaN_3 buffer before binding assay.

TABLE III

EFFECTS OF DIVALENT CATIONS AND CHELATING AGENTS ON [^{125}I]αBuTx AND [^{3}H]QNB BINDING TO BRAIN MEMBRANES

See Table II for control values and treatment protocol. Greater than 95% of protein was recovered after treatment.

Treatment	Concentration (M)	[^{125}I]αBuTx (% of control)	[^{3}H]QNB (% of control)
$CaCl_2$	$1 \cdot 10^{-3}$	68.0	89.6
	$1 \cdot 10^{-4}$	74.3	90.1
$SrCl_2$	$1 \cdot 10^{-3}$	121.5	108.6
	$1 \cdot 10^{-4}$	115.0	89.5
Na_2EDTA	$1 \cdot 10^{-2}$	106.3	111.8
	$1 \cdot 10^{-3}$	106.0	94.1
EGTA	$1 \cdot 10^{-2}$	86.9	87.7
	$1 \cdot 10^{-3}$	95.6	89.2

and sulfhydryl groups, has yielded considerable information about the importance of these groups in the functioning of several types of neurotransmitter receptor [19—22]. Table IV shows the effects of several of these agents on [^{125}I]αBuTx and [^{3}H]QNB binding to brain membranes. The disulfide reducing agents, β-mercaptoethanol and dithiothreitol, an alkylating agent, *N*-ethylmaleimide, and sodium metabisulfite, an agent believed to produce heterolytic cleavage of protein disulfide bonds [23], had little effect on [^{3}H]QNB binding. In contrast, dithiothreitol ($1 \cdot 10^{-2}$ M)-pretreated membranes showed a marked increase (40%) in the amount of [^{125}I]αBuTx binding. This enhancement in toxin binding was due entirely to an increase in the number of sites since the K_D value for [^{125}I]αBuTx binding determined by Scatchard analysis of treated membranes (data not shown) was identical to that for untreated membranes ($K_D = 1 \cdot 10^{-9}$ M). All specific [^{125}I]αBuTx binding in either dithiothreitol-treated or untreated membranes could be completely blocked by $1 \cdot 10^{-4}$ M (+)-tubocurarine. In contrast to the lack of effect produced by *N*-ethylmaleimide alkylation, the amount of both [^{125}I]αBuTx and [^{3}H]QNB binding was decreased 65 and 31%, respectively, when the membranes were pretreated with dithiothreitol and then alkylated with *N*-ethylmaleimide.

The bottom half of Table IV shows the results of disulfide and sulfhydryl modification when excess reagents are not removed prior to binding assay. Again, a large increase in the amount of [^{125}I]αBuTx binding was observed as a result of dithiothreitol treatment. When membranes were treated with the sulfhydryl modifying reagents, dithio-bis(2-nitrobenzoic acid), a sulfhydryl oxidizing agent which may promote reformation of disulfide bonds, or *p*-chloromercuribenzoate, an organomercurial with high affinity and specificity for sulfhydryl groups, a 17 and 23% reduction in [^{125}I]αBuTx binding was observed. Both these reagents could reverse the dithiothreitol-induced increase in [^{125}I]-αBuTx binding when added after prior reduction of the membranes with dithiothreitol. In fact, dithiothreitol pretreatment followed by dithio-bis(2-nitrobenzoic acid) or *p*-chloromercuribenzoate resulted in considerably more

TABLE IV

EFFECTS OF DISULFIDE AND SULFHYDRYL REAGENTS ON [^{125}I]αBuTx AND [^{3}H]QNB BINDING TO BRAIN MEMBRANES

See Table II for control values and recovery, and for treatment protocol. In B, aliquots of membrane (0.3 mg) were incubated 15 min in 0.2 ml Tris, NaCl, NaN_3 buffer at the indicated concentration of reagent. [^{125}I]αBuTx or [^{3}H]QNB was then added directly to the treatment tube and binding assayed as described in Materials and Methods

	Concentration (M)	[^{125}I]αBuTx (% of control)	[^{3}H]QNB (% of control)
(A) Reagents removed prior to assay			
β-Mercaptoethanol	$1 \cdot 10^{-2}$	93.7	103.3
	$1 \cdot 10^{-3}$	94.1	109.3
	$1 \cdot 10^{-4}$		101.3
Dithiothreitol	$1 \cdot 10^{-2}$	140.0	103.2
	$1 \cdot 10^{-3}$	93.7	98.3
	$1 \cdot 10^{-4}$	89.5	105.0
Sodium metabisulfite	$1 \cdot 10^{-3}$	107.9	82.7
N-Ethylmaleimide	$1 \cdot 10^{-2}$	99.6	103.4
	$1 \cdot 10^{-3}$	112.2	110.0
	$1 \cdot 10^{-4}$	100.2	112.0
Dithiothreitol, then *N*-Ethylmaleimide *	$1 \cdot 10^{-3}$, $1 \cdot 10^{-3}$	35.0	68.7
(B) Reagents present during assay			
Dithiothreitol	$1 \cdot 10^{-4}$	160.7	112.5
Dithio-bis(2-nitro-benzoic acid)	$1 \cdot 10^{-3}$	82.6	—
p-Chloromercuribenzoate	$1 \cdot 10^{-3}$	77.2	—
Dithiothreitol, then	$1 \cdot 10^{-4}$, $1 \cdot 10^{-3}$	31.6	—
dithio-bis(2-nitro-benzoic acid) **	$1 \cdot 10^{-4}$, $1 \cdot 10^{-2}$	24.6	—
Dithiothreitol, then	$1 \cdot 10^{-4}$, $1 \cdot 10^{-3}$	28.0	—
p-chloromercuribenzoate **	$1 \cdot 10^{-4}$, $1 \cdot 10^{-3}$	13.0	—

* Excess dithiothreitol removed by centrifugation before treatment for additional 30 min with *N*-ethylmaleimide followed by a subsequent centrifugation.

** Dithiothreitol treatment for 15 min followed by dithio-bis(2-nitrobenzoic acid) or *p*-chloromercuribenzoate at the indicated concentration for an additional 15 min. Samples were then assayed as described in Materials and Methods.

inhibition of [^{125}I]αBuTx binding (greater than 70%) than either agent alone (less than 23%).

The amount of [^{125}I]αBuTx binding as a function of dithiothreitol concentration is presented in Fig. 1. Higher concentrations of dithiothreitol resulted in an apparent decrease in toxin binding. This effect is most likely explained by the inactivation of [^{125}I]αBuTx rather than a direct effect on the membrane binding sites. αBuTx is a polypeptide toxin with five disulfide bonds which will compete with the membrane disulfides for available dithiothreitol. Thus, there was a 33% reduction in toxin binding (presumably due to inactivation of the toxin) when 0.05 mM dithiothreitol and toxin were added simultaneously to the assay mixture in contrast to a 40% increase in toxin binding when the membranes were pretreated for 30 min with 0.05 mM dithiothreitol prior to addi-

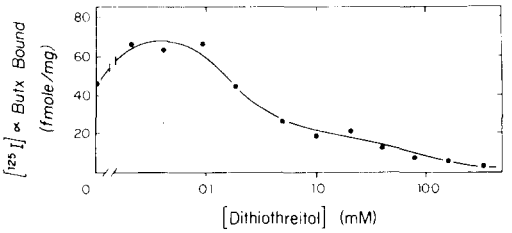

Fig. 1. Effect of dithiothreitol on [^{125}I]αBuTx binding to brain membranes. Triplicate samples (0.3 mg) were preincubated at room temperature with the indicated concentration of dithiothreitol in 0.2 ml of Tris, NaCl, NaN_3 buffer for 30 min before addition of [^{125}I]αBuTx to a final concentration of $4 \cdot 10^{-9}$ M for the filled circles. The open circle represents the simultaneous addition of dithiothreitol and [^{125}I]-αBuTx.

tion of [^{125}I]αBuTx (see Fig. 1).

The effects of various enzymatic treatments on [^{125}I]αBuTx and [^{3}H]QNB binding are presented in Table V. Binding of both ligands appeared relatively stable to DNAase I, bacterial protease and neuroaminidase digestion. Toxin binding was reduced 19% by trypsin digestion while [^{3}H]QNB binding was not affected under identical conditions. The amount of binding for both ligands

TABLE V

EFFECT OF VARIOUS ENZYMES ON [^{125}I]αBuTx AND [^{3}H]QNB BINDING TO BRAIN MEMBRANES

An aliquot of membrane (4—10 mg) was incubated 30 min at 37°C with the indicated concentration of enzyme in 6 ml of Tris, NaCl, NaN_3 buffer. The membranes were then centrifuged as described in the text. See Table I for control values and recovery

Enzyme	Concentration (M)	[^{125}I]αBuTx (% of control)	[^{3}H]QNB (% of control)
DNAse I	$1 \cdot 10^{-6}$	98.1	99.9
	$1 \cdot 10^{-7}$	101.6	96.1
	$1 \cdot 10^{-8}$	109.1	92.6
Trypsin	$1 \cdot 10^{-6}$	81.0	100.0
	$1 \cdot 10^{-7}$	98.1	107.0
Protease (*Bacillus thermoproteolyticus*)	(0.5 mg/ml)	97.3	95.6
Neuraminidase (*Vibro cholerae*)	(1 mg/ml)	99.8	91.9
Phospholipase A_2 (*Crotalus adamantus*) + $CaCl_2$ ($1 \cdot 10^{-3}$ M)	$1 \cdot 10^{-6}$	45.6	42.3
Phospholipase A_2 (no $CaCl_2$)	$1 \cdot 10^{-6}$	103.2	97.8
β-Bungarotoxin + $CaCl_2$ ($1 \cdot 10^{-3}$ M)	$1 \cdot 10^{-6}$	15.5	6.02
	$1 \cdot 10^{-7}$	49.5	48
	$1 \cdot 10^{-8}$	85	81
β-Bungarotoxin (no $CaCl_2$)	$1 \cdot 10^{-6}$	13.5	4.25

was reduced by phospholipase A_2 treatment and β-bungarotoxin, a toxic component of *B. multicinctus* venom with known phospholipase A_2 activity [24]. The reduction in binding produced by *C. adamantus* phospholipase A_2 was dependent on exogenously added Ca^{2+} while the effect produced by β-bungarotoxin was not. The reduction in [^{3}H]QNB binding could be prevented by adding EGTA ($1 \cdot 10^{-2}$ M) to chelate tightly bound endogenous Ca^{2+} in the membranes, but the reduction in [^{125}I]αBuTx binding could not be prevented under identical conditions (Salvaterra, P.M., unpublished results).

Chemical and enzymatic treatment of brain membranes not only results in the modification of binding for putative neurotransmitter receptor antagonists but may also produce changes in putative agonist binding properties as well. Since agonists generally bind to receptors with lower affinity than antagonists, the former are difficult to study in direct binding assays. Agonist binding can be studied indirectly, however, by observing its concentration-dependent ability to prevent antagonist binding. Fig. 2 shows the dose-dependent effects of nicotine, a nicotinic colinergic agonist, in inhibiting [^{125}I]αBuTx binding to membrane binding sites. Little or no change in nicotine-dependent inhibition of [^{125}I]αBuTx binding was observed for the agents shown in Fig. 2. In Fig. 3, there was an apparent shift to the right for the nicotine inhibition curve when membranes were treated with the disulfide reducing agents, β-mercaptoethanol and dithiothreitol, or with removal of tightly bound endogenous divalent cations, presumably Ca^{2+}, by EDTA or EGTA. These results can be interpreted as a decrease in the binding affinity of nicotine for reduced or cation-depleted membranes. The effect appeared limited to agonist binding, since a similar shift in inhibitory potency was not observed for the classical nicotinic acetylcholine receptor antagonist, (+)-tubocurarine, as shown in Fig. 4. The ability of Ca^{2+} removal or dithiothreitol treatment to lower the potency of nicotine in inhibiting [^{125}I]αBuTx appeared to be at least partially additive as shown in Table V.

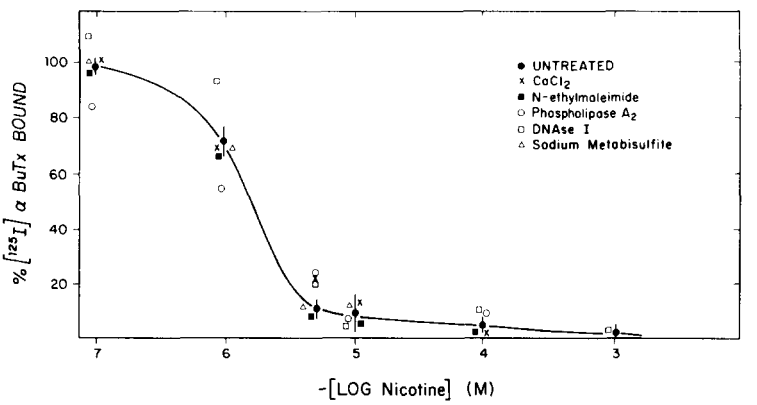

Fig. 2. Effect of various chemical and enzymatic treatments on nicotine inhibition of [^{125}I]αBuTx binding to brain membranes. Samples were treated as in the footnotes to Tables III—V at the highest concentration indicated. Excess reagents or enzymes were removed prior to binding assay by centrifugation. Samples were assayed by preincubation for 30 min with nicotine before addition of [^{125}I]αBuTx to a final concentration of $1 \cdot 10^{-9}$ M. Control samples with no nicotine were included for comparison of each treatment as well as untreated membranes. Values shown are means of triplicates repeated at least once with standard deviations shown for untrated membranes.

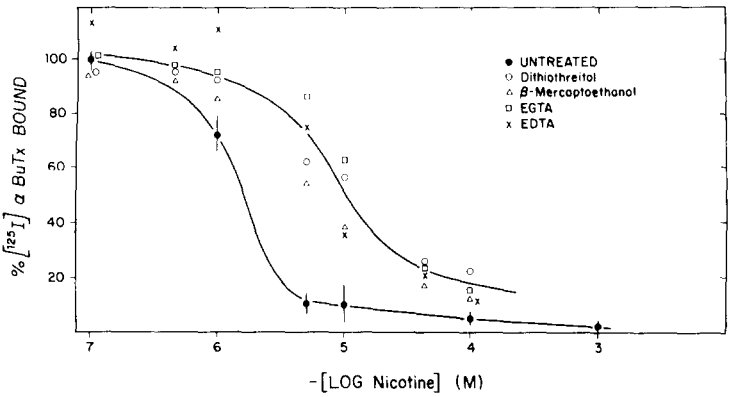

Fig. 3. Effect of treatment with disulfide reducing agents and divalent metal cation chelating agents on nicotine inhibition of [^{125}I]αBuTx binding to brain membranes. Samples were treated as described in footnotes to Tables III and IV at the highest concentration indicated. See Fig. 2 for additional details.

A 40—50% decrease in toxin binding was seen when EGTA- or dithiothreitol-treated membranes were incubated with $1 \cdot 10^{-5}$ M nicotine, while the inhibition was only 27% when membranes were treated with both EGTA and dithiothreitol.

Inhibition of [^{3}H]QNB binding after various treatments was studied as a function of the cholinergic agonist, carbamylcholine. Most of the chemical and enzymatic treatments resulted in little or no change in carbamylcholine binding (Fig. 5). Alkylation of membranes with *N*-ethylmaleimide, however, resulted in a shift to the left for the carbamylcholine vs. [^{3}H]QNB binding curve (Fig. 6). This shift can be interpreted as an increase in carbamylcholine affinity, since *N*-ethylmaleimide treatment alone caused little or no change in [^{3}H]QNB binding (see Table IV).

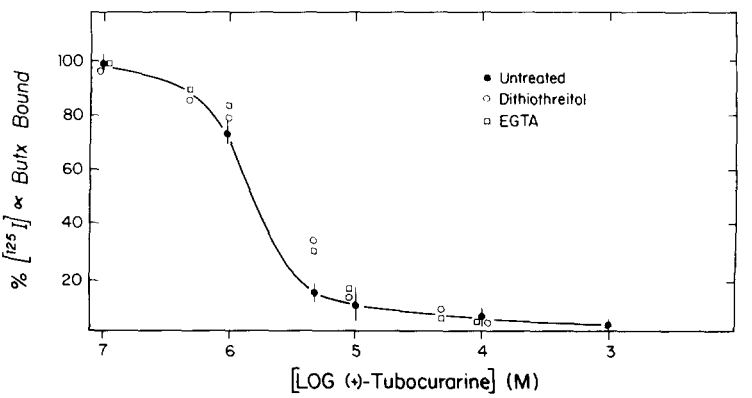

Fig. 4. Effect of treatment with dithiothreitol and EGTA on (+)-tubocurarine inhibition of [^{125}I]αBuTx binding to brain membranes. Samples were treated as described in footnotes to Tables III and IV at the highest concentration indicated. Samples were preincubated 30 min with (+)-tubocurarine before addition of [^{125}I]αBuTx to a final concentration of $1 \cdot 10^{-9}$ M. See Fig. 2 for additional details.

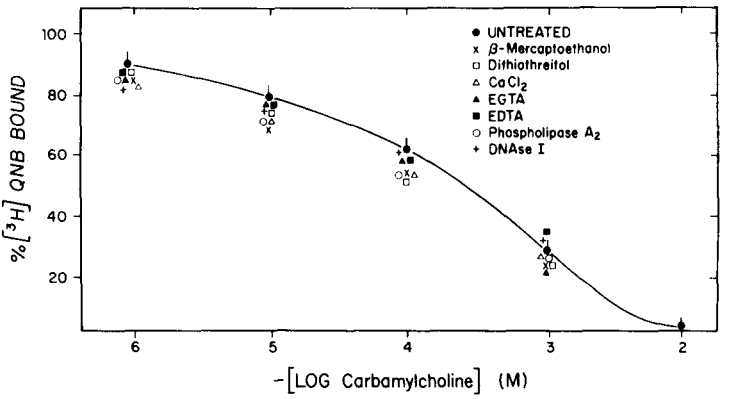

Fig. 5. Effect of various chemical and enzymatic treatments on carbamylcholine inhibition of [^{3}H]QNB binding to brain membranes. Samples were treated as described in the footnotes to Table III—V at the highest concentration indicated. Excess reagents or enzymes were removed prior to binding assay by centrifugation. Samples were assayed by preincubation for 30 min with carbamylcholine before addition of [^{3}H]QNB to a final concentration of $1 \cdot 10^{-9}$ M. Control samples with no carbamylcholine were included for comparison of each treatment, as well as untreated membranes. Values shown are means of triplicate determinations repeated at least once with standard deviations shown for untreated membranes.

Discussion

Both [^{125}I]αBuTx and [^{3}H]QNB binding appeared to be stable to the various forms of physical perturbation investigated. Stability to repeated freeze-thawing, as well as vigorous mechanical shearing, indicates a tight coupling of the ligand sites to the membrane fraction and resistance to denaturation following these treatments. Most likely, they are integral membrane proteins. The binding sites must also be protected from attack by endogenous enzyme activity, since little change in the amount of binding was detected in samples prepared at 22°C. This preparation protocol would be expected to result in considerable enzymatic autolysis of any unprotected proteins. In addition, the membrane

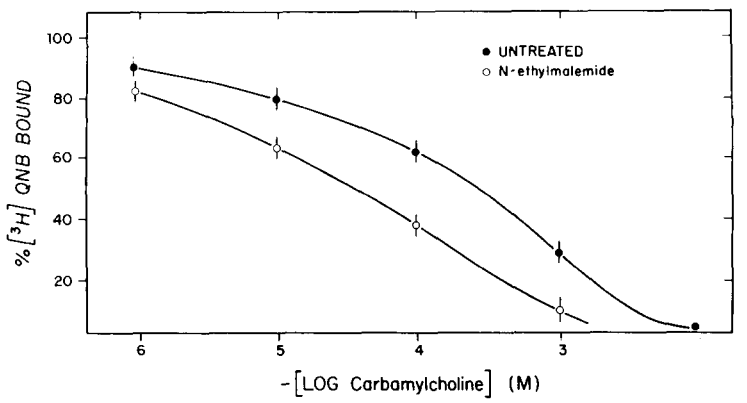

Fig. 6. Effect of *N*-ethylmaleimide treatment on carbamylcholine inhibition of [^{3}H]QNB binding to brain membranes. Samples were treated as described in footnote to Table III. Excess reagent was removed by centrifugation prior to binding assay. See Fig. 5 for additional details.

binding was also quite stable to exogenously added proteases (Table V) although the detergent-solubilized [^{125}I]αBuTx binding sites from rat brain are easily destroyed by trypsin, indicating their essential protein nature.

Binding of both [^{125}I]αBuTx and [^{3}H]QNB was sensitive to phospholipase A_2 and β-bungarotoxin treatment, indicating the possible involvement of phospholipid. It is not known whether the effect is a direct one resulting from hydrolysis of an essential phospholipid component of the receptor or an indirect effect on binding produced by lysophospholipid inhibition of the binding assay as recently shown for [^{3}H]QNB [25]. The binding of both [^{125}I]αBuTx and [^{3}H]QNB was more sensitive to treatment with β-bungarotoxin, a neurotoxic component of *B. multicinctus* venom with known Ca^{2+}-dependent phospholipase activity [24], than *Crotalus* phospholipase added at an equimolar concentration. The inhibition is not dependent on exogenously added Ca^{2+}, and only the reduction in [^{3}H]QNB binding can be prevented by chelating tightly bound endogenous Ca^{2+} with EGTA prior to β-bungarotoxin incubation. Further studies are currently underway to describe the effects of β-bungarotoxin on [^{125}I]-α-BuTx and [^{3}H]QNB binding to rat brain membranes.

The decrease in [^{3}H]QNB binding after digitonin treatment (Table II) may not be due to inactivation but rather to solubilization of the muscarinic acetylcholine receptor as recently reported [26,27].

The relative stability of both [^{125}I]αBuTx and [^{3}H]QNB binding to glutaraldehyde treatment may prove useful in further attempts to isolate synaptic junctions with preserved neurotransmitter receptor activity. Methods currently available for isolation of synaptic junctions from synaptic plasma membrane fractions require detergent treatment [28—30] and result in an inhibition and/or solubilization of ligand binding sites. Glutaraldehyde pretreatment may cross-link receptors with each other or other membrane proteins and possibly prevent their subsequent solubilization and/or inactivation during detergent treatment, and thus permit evaluation of ligand binding in resulting synaptic junction fractions.

Treatment of membranes with physiological concentrations of Ca^{2+} resulted in an apparent inhibition of antagonist binding (Table III) while Sr^{2+} seemed to result in a slight increase in [^{125}I]αBuTx binding. The decrease in [^{125}I]αBuTx binding was a relatively stable change, since the excess Ca^{2+} was removed by centrifugation before the binding assay in contrast to our earlier studies where added Ca^{2+} was present during the binding assay [18]. Sr^{2+} may prevent the action of Ca^{2+}-dependent endogenous phospholipases or proteases which may tend to lower toxin binding. Although the removal of membrane-bound Ca^{2+} with EGTA resulted in a slight reduction of [^{125}I]αBuTx, as well as [^{3}H]QNB binding, there was a more pronounced effect on agonist binding (see below).

The action of disulfide and sulfhydryl reagents on antagonist binding (Table IV) may indicate involvement of these groups in both [^{125}I]αBuTx and [^{3}H]-QNB binding. [^{3}H]QNB binding appeared less sensitive to most of the reagents tested with only a slight decrease (17%) after treatment with sodium metabisulfite. Although *N*-ethylmaleimide alkylation or dithiothreitol reduction produced little or no effect on [^{3}H]QNB binding, there was a significant (31%) reduction in [^{3}H]QNB binding when membranes that were reduced with dithiothreitol were subsequently alkylated with *N*-ethylmaleimide. This result may

indicate a reducible disulfide group involved in [^{3}H]QNB binding. Our results are in agreement with the recent studies of Aronstram et al. [31] where disulfide and sulfhydryl reagent effects on [^{3}H]QNB binding could be modified by coincubating the membranes with muscarinic agonists or antagonists. These experiments [31] indicate that cholinergic ligands can exert conformational control of the disulfide and sulfhydryl groups undergoing modification. A somewhat different pattern of disulfide and sulfhydryl group modification on [^{3}H]QNB binding has been presented by Hedlund and Bartfi [32]. They have observed direct effects of *N*-ethylmaleimide and dithiothreitol on [^{3}H]QNB binding and attribute the apparent discrepancies to the results of Aronstram et al. [31] to different buffer conditions.

[^{125}I]αBuTx binding was also sensitive to the state of the disulfide and sulfhydryl groups on the membranes. Physiological studies of peripheral nicotinic receptors have implicated involvement of these protein groups in functional states of the receptor [19—23]. When membranes were pretreated with the disulfide reducing agent, dithiothreitol, we observed a significant increase in the number of detectable [^{125}I]αBuTx binding sites. This phenomenon is a true unmasking of otherwise undetected sites, since no change in affinity for [^{125}I]-αBuTx was observed (Scatchard plot). These additional sites are also pharmacologically similar to untreated sites, since all specific toxin binding is blocked by preincubation with (+)-tubocurarine. The increase in toxin binding sites after dithiothreitol reduction appeared to be a transient phenomenon. Removal of dithiothreitol by centrifugation prior to toxin binding assay resulted in a 40% increase in toxin sites and required $1 \cdot 10^{-2}$ M dithiothreitol. In contrast, when dithiothreitol was not removed prior to toxin binding assay, a 60% increase resulted at only $1 \cdot 10^{-4}$ M dithiothreitol. The time required for removal of dithiothreitol during centrifugation may result in a reoxidation of the critical disulfide bond(s) which, when reduced, result in increased toxin binding. In a recent report, Lukas et al. [34] observed no effect of dithiothreitol on [^{125}I]αBuTx binding to rat brain membranes, and the difference between their results [34] and the results presented here is most likely due to the different treatment protocols. Experiments in this paper used purified membranes, while they used a crude homogenate which may contain more non-relevant disulfide groups which may interact with dithiothreitol and thus mask any direct effect of disulfide bond reduction on [^{125}I]αBuTx binding.

An inhibitory effect on [^{125}I]αBuTx binding was observed when membranes were treated with dithio-bis(2-nitrobenzoic acid) and *p*-chloromercuribenzoate both before and after dithiothreitol reduction (Table IV). The decrease in binding is greater after reduction of the membranes with dithiothreitol indicating the involvement of both disulfide and sulfhydryl groups in [^{125}I]αBuTx binding.

Extensive studies of disulfide and sulfhydryl group modification have been related to the physiological function of peripheral nicotinic acetylcholine receptors. In muscle preparations, dithiothreitol reduction results in a decreased responsiveness of muscle to certain agonists [21,22], while heterolytic cleavage of disulfide bonds results in an increased responsiveness [33] indicating disulfide bond participation in different functional states of the receptor. Physiological results which have been interpreted in terms of reduced

agonist affinity in dithiothreitol-treated tissue are consistent with our observation of reduced in vitro agonist binding affinity (see Fig. 4). The affinity change appears limited to agonist binding, since no comparable change was observed for the antagonist (+)-tubocurarine. This result is in essential agreement with that reported by Lukas et al. [34] for carbamylcholine inhibition of αBuTx binding to crude brain membranes and extended to similar observations for *Torpedo* nicotinic acetylcholine receptors [35].

A similar change in nicotinic agonist affinity was observed for membranes depleted of divalent cations by EGTA and EDTA. Although qualitatively similar to the effect seen with reduction by dithiothreitol, the decrease in affinity by cation depletion may occur through a different mechanism (conformational state change?), since the effects of dithiothreitol and EGTA were observed to be additive (see Table VI). Several physiological studies have suggested a role for Ca^{2+} in modifying receptor function [36,37], and in vitro studies with well characterized nicotinic receptors from electroplax have indicated a competitive inhibition of Ca^{2+} and cholinergic agonists [38,39].

The effects of chemical modification on muscarinic receptor function are not as extensively studied as nicotinic receptors. Sulfhydryl group modification has been reported to result in changes in the functional properties of muscarinic systems in smooth muscle [40,41]. The *N*-ethylmaleimide alkylation-induced affinity change we have observed for carbamylcholine binding is in essential agreement with that reported by Aronstram et al. [31] and confirmed by Hedlund and Bartfai [32].

While it is tempting to relate in vitro binding results to in vivo receptor function, such a synthesis for central receptors appears premature. Further physiological experiments are necessary to prove that [^{125}I]αBuTx and [^{3}H]QNB do indeed label relevant cholinergic receptors. The results presented in this study, particularly with regard to changes in agonist binding affinity as a result of

TABLE VI

EFFECT OF DITHIOTHREITOL AND EGTA TREATMENT ON THE ABILITY OF NICOTINE TO INHIBIT [^{125}I]αBuTx BINDING TO BRAIN MEMBRANES

Values represent means of triplicate determinations with standard errors of less than 10%. Control value for toxin binding at 1 nM [^{125}I]αBuTx was 28 fmol/mg

Treatment	Nicotine concentration (M)	[^{125}I]αBuTx binding (% of control)
None	0	100
None	$1 \cdot 10^{-5}$ *	12.0
Dithiothreitol ($1 \cdot 10^{-2}$ M) **	$1 \cdot 10^{-5}$ *	60.1
EGTA ($1 \cdot 10^{-3}$ M) **	$1 \cdot 10^{-5}$	49.1
Dithiothreitol ($1 \cdot 10^{-2}$ M), then EGTA ($1 \cdot 10^{-3}$ M) ***	$1 \cdot 10^{-5}$	73.3

* Samples preincubated 30 min at room temperature with nicotine before addition of $1 \cdot 10^{-9}$ M [^{125}I] αBuTx.

** Membrane samples (10 mg) were treated for 30 min at room temperature in 1 ml of Tris, NaCl, NaN_3 buffer. 6 ml of Tris, NaCl, NaN_3 buffer were then added, and samples were pelleted at 150 000 × *g* · 30 min.

*** The sample was incubated an additional 30 min with EGTA after the initial treatment with dithiothreitol as described above.

disulfide and/or sulfhydryl group modification, would support such an identification.

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