BIOCHEMICAL STUDIES OF THE NICOTINIC ACETYLCHOLINE RECEPTOR FROM *TORPEDO CALIFORNICA* ELECTROPLAX

Thesis by

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ABSTRACT

The ligand binding properties of the acetylcholine (AcCh) receptor have been investigated using radioactive cholinergic ligands, radiolabelled α-neurotoxins, and fluorescent decamethonium analogues. The radioactive cholinergic ligands appear to bind with high affinity to half as many sites as α-Bungarotoxin (α-BuTx). One possible interpretation of these data is that two distinct classes of sites are present in equal populations. The results of kinetic experiments conducted in this laboratory have shown that the receptor binds α-BuTx with a five fold greater second order rate constant and binds cholinergic ligands with 2-7 fold lower affinity to the "fast" than to the "slow" site.

The binding properties of a series of synthetic bis-3-amino-pyridinium-1,n-alkane fluorescent decamethonium analogues to the purified receptor were investigated. The probes bind with substantially greater affinity to the "slow" than to the "fast" site, and therefore served as useful tools to map the ligand binding topography of the "slow" site of the receptor. This site binds the probes with increasing affinity as the methylene bridge length (n) increases from 4 to 12 carbons and decreases from 18 to 16 carbons. One possible interpretation of these data is that when the "slow" site binds a bifunctional probe such as bis-3-aminopyridinium-1,14-tetradecane, both ends of the probe interact strongly with the receptor. Therefore the "slow" site of the receptor may be composed

of at least two subsites.

Recently published evidence indicates that the venom of the krait Bungarus caeruleus contains unique toxic proteins which affect the function of the AcCh receptor in vivo. Therefore we undertook the fractionation and purification of various proteins from this venom in order to examine their use as probes of the AcCh receptor. Two of the basic components were highly neurotoxic in mice and had significant levels of phospholipase-A activity. These components appear to be similar to the presynaptic neurotoxin β-BuTx. Two other basic components were toxic in mice and also reduced the rate of α-BuTx binding to the purified acetylcholine receptor. These components appear to be similar to the postsynaptic neurotoxin α-BuTx. Two acidic components display A-type phospholipase activity and perturb the carbamylcholine binding properties of receptor enriched membrane preparations. These data indicate that the fatty acids and/or lysophospholipids released by endogenous or exogenous phospholipase A may affect the receptor conformation such that the receptor binds carbamylcholine with greater affinity.

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ABBREVIATIONS

AcCh	-acetylcholine
BuTx	-Bungarotoxin
CoTx	-Cobratoxin
Carb	-carbamylcholine
Deca	-decamethonium
HTx	-histionicotoxin
d-TC	-d-tubocurarine
MBTA	-4-N-maleimido-α-benzyltrimethylammonium
DFP	-diisopropylfluorophosphate
BAP	-bis-3-aminopyridinium-1,4-butane
HAP	-bis-3-aminopyridinium-1,6-hexane
OAP	-bis-3-aminopyridinium-1,8-octane
DAP	-bis-3-aminopyridinium-1,10-decane
DoDAP	-bis-3-aminopyridinium-1,12-dodecane
TetraDAP	-bis-3-aminopyridinium-1,14-tetradecane
HexaDAP	-bis-3-aminopyridinium-1,16-hexadecane
OctaDAP	-bis-3-aminopyridinium-1,18-octadecane
SDS	-sodium dodecylsulfate
Tris	-trihydroxymethylaminomethane
HEPES	-N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
DEAE	-diethylaminoethyl
DTT	-dithiothreitol

DTNB	-5,5'-dithiobis-(2-nitrobenzoic acid)
EGTA	-ethyleneglycol-bis-(β-aminoethylether)-N,N'-tetra-acetic acid
QAE	-diethyl-(2-hydroxy-propyl)-aminoethyl
CM	-carboxymethyl
MW	-molecular weight
Kd	-equilibrium dissociation constant
i.p.	-intraperitoneal

INTRODUCTION

The acetylcholine receptor is defined as that component of the postsynaptic membrane at the neuromuscular junction which binds neurotransmitter and causes a transient depolarization at the end plate region. If this transient depolarization exceeds threshold level, an electrical impulse is propagated throughout the muscle cell membrane and this normally leads to muscle fiber contraction. The AcCh receptor essentially transduces a chemical signal, acetylcholine, into an electrical response.

Over the past several decades physiological experiments have yielded a wealth of information concerning the functional aspects of the neuromuscular junction. However, in order to determine the mechanism by which the receptor transduces the chemical signal into an electrical response, an increased understanding of the AcCh receptor is needed at the molecular level. The research presented here focuses on the biochemical properties of the acetylcholine receptor isolated from Torpedo californica electroplax.

The electric organs (electroplax) of the elasmobrach ray Torpedo and the freshwater eel Electrophorus bear close functional similarities to the neuromuscular junction. In both systems AcCh is the chemical transmitter and the AcCh receptor transduces the chemical signal into an electrical response. In the case of the neuromuscular junction this leads to the contraction of the muscle fiber whereas in the case of a cell (electroplaque) from the electric organ this simply leads to discharge of electric current. The

electroplaque has few if any contractile filaments (1). Since the density of cholinergic receptors per gram wet tissue in Torpedo electroplax is at least an order of magnitude greater than that of Electrophorus electroplax, and two orders of magnitude greater than that of the mouse or rat diaphragm (2), it represents an excellent source of receptor for biochemical studies. As a background to the biochemical studies presented here the following topics are discussed:

1) chemical transmission of AcCh at the motor nerve terminal,
2) pharmacological agents which affect neuromuscular transmission,
3) a ligand binding model of the pharmacological data, and
4) biochemical properties of the isolated nicotinic acetylcholine receptor.

1) Chemical Transmission of AcCh at the Motor Nerve Terminal:

At the motor end plate impulses are propagated from nerve axons to skeletal muscles via the chemical transmitter, acetylcholine (3). AcCh is synthesized in the motor nerve terminal and stored in specialized structures called synaptic vesicles. It is estimated that an average synaptic vesicle contains approximately 10^4 molecules of AcCh. The release of the contents of one vesicle constitutes a

quantum event. When a nerve impulse reaches the motor nerve terminal, hundreds of quanta of AcCh are released synchronously (1). The release process is dependent upon the entry of Ca^{++} ions into the presynaptic terminal (4) and the exocytosis of AcCh-containing vesicles at specialized sites on the presynaptic membrane called active zones (5). The released AcCh diffuses across the approximately 500 Å gap, or synapse, between the nerve terminal and the muscle fiber surface, whereupon it binds to receptors embedded in the postsynaptic membrane (6). The postsynaptic membrane is specialized with deep invaginations called junctional folds. At the crests of these folds the AcCh receptors are clustered in high density (7). The concentration of neurotransmitter in the synaptic cleft after depolarization of the presynaptic terminal is approximately 300 μM; the concentration of AcCh receptors at the crest of the junctional folds is calculated to be 1000 μM (1). It is estimated that 60% of the transmitter released binds to and activates the postsynaptic AcCh receptors (1). Binding of AcCh to its receptor results in a permeability increase at the end plate region to Na^+ and K^+ which subsequently causes a transient depolarization of the postsynaptic membrane at the end plate region (8).

The Na^+ and K^+ ion permeability increase at the endplate region occurs within 0.3 msec after AcCh is released from the presynaptic nerve terminal (1). Karlin (9) calculates that 10^4 cations per millisecond per receptor pass through the postsynaptic membrane. Since this flux is too rapid to be mediated by simple

diffusion across the membrane or by an ion-carrier which traverses the membrane, he postulates that Na^+ and K^+ pass through an ion channel functionally regulated by the AcCh receptor. This channel is open for 1 msec when the receptor is activated by bound AcCh (10,11). When the channel closes, transmitter is released from the receptor. Within 2 msec the free AcCh is hydrolyzed by esterase which is distributed throughout the junctional folds (1). The speed at which AcCh is released, bound, and inactivated makes it possible for the process of neuromuscular transmission to be repeated several hundred times a second.

2) Pharmacological Agents Which Affect Neuromuscular Transmission.

Neuromuscular transmission is blocked if: a) insufficient quantities of AcCh are released into the synaptic cleft after nerve stimulation, b) the cholinergic ligand binding site is occupied by an antagonist, or c) AcCh binding to the receptor does not lead to increased cation permeability at the endplate region. When a severe neuromuscular block develops, vertebrate respiratory muscles become paralyzed, resulting in death by asphyxiation (8).

Several highly potent presynaptic toxins are known. Botulinumtoxin inhibits the fusion of synaptic vesicles with the presynaptic membrane (12). β-BuTx, which is purified from the venom of the krait Bungarus multicinctus, is a basic polypeptide which has a molecular weight of 21,000 daltons (13). The mechanism by

which β-BuTx inhibits transmitter release is not known (14).

Several specific postsynaptic neurotoxins have been characterized also. α-BuTx, purified from the venom of Bungarus multicinctus, is a basic polypeptide of MW 8,000 daltons (15,16). Since this toxin blocks the depolarization of the Electrophorus electroplaque caused by carbamylcholine (a cholinergic agonist which is slowly hydrolyzed by AcCh esterase) in an essentially irreversible manner, Changeux et al. (17) proposed that this toxin binds to and blocks the cholinergic ligand binding site. α-CoTx, which is purified from the venom of the cobra Naja naja atra, is a basic polypeptide of MW 6,000 daltons (18). Also, this toxin binds to the cholinergic ligand binding site with high affinity.

Recently, histrionicotoxin was purified from the skin of the frog Dendrobates histrionicus. This toxin is postulated to interfere with the opening of the receptor mediated ion channel (19). Kato and Changeux performed intracellular recordings on an Electrophorus electroplaque (20) and observed that the depolarization produced by 10 μM carbamylcholine was reduced by 50% in the presence of 0.5 μM histrionicotoxin (HTx). Also, they observed that the relative amount of ^{3}H-AcCh bound to Torpedo membrane preparations enriched for the AcCh receptor was reduced by 50% in the presence of 200 μM HTx. Since significantly lower concentrations of HTx are required to inhibit the depolarization of the Electrophorus cell than to inhibit binding of AcCh to the Torpedo

receptor, it is quite possible that HTx binds to and blocks the receptor mediated ion channel.

Numerous cholinolytic ligands are known which bind to the cholinergic ligand binding site and antagonize the depolarization produced by agonists such as AcCh or Carb. d-Tubocurarine (d-TC) is an alkaloid found in plants of the genus Strychnos. Bovet (21) investigated the pharmacology of d-TC extensively and as a result synthesized the antagonists flaxedil and hexamethonium as well as the depolarizing antagonists succinylcholine and decamethonium. The latter two compounds depolarize the Electrophorus electroplaque when applied singly but also antagonize the depolarization produced by Carb (2). The chemical structures of the various cholinergic ligands are shown in figure 1. These pharmacological agents act in a similar manner on the Torpedo electroplaque (22), the Electrophorus electroplaque (2), and the end plate region of frog sartorius muscle (2).

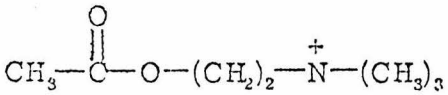
3) A Ligand Binding Model of the Pharmacological Data.

Rang (2) proposed a two state model to explain the effects of agonists, antagonists, and depolarizing antagonists. If the receptor is in the R conformation the receptor mediated ion channel (ion translocation device) is closed, whereas if the receptor is in the R' conformation the ion translocation device is open. If a cholinergic ligand binds to the R' conformation with high affinity

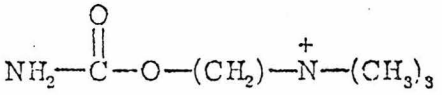
Figure 1.

Chemical structures of various cholinergic ligands.

Agonists

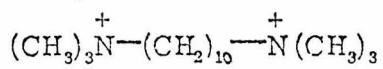


Acetylcholine

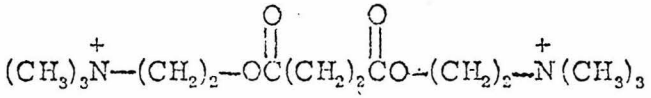


Carbamylcholine

Depolarizing Agonists

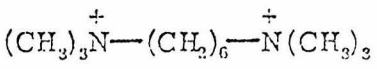


Decamethonium

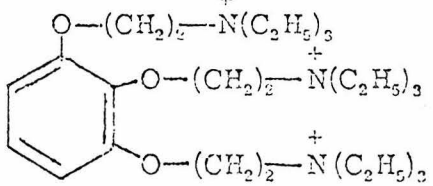


Succinylcholine

Antagonists

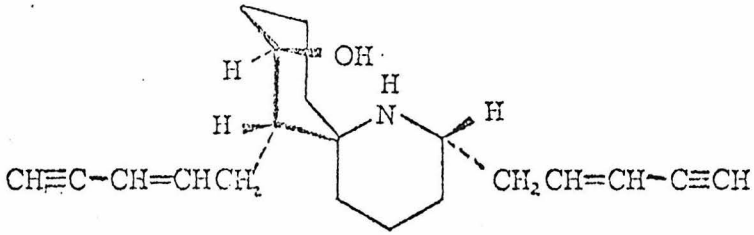


Hexamethonium

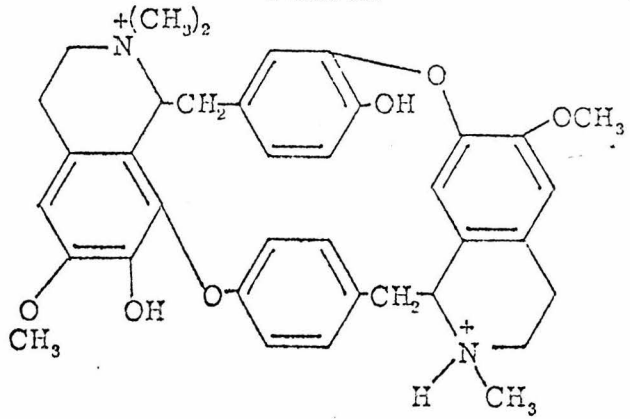


Flaxedil

Possible Blocker of Ion Translocation Device



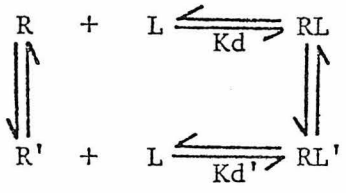
Histrionicotoxin



d-Tubocurarine

α- Bungarotoxin, α - Cobratoxin

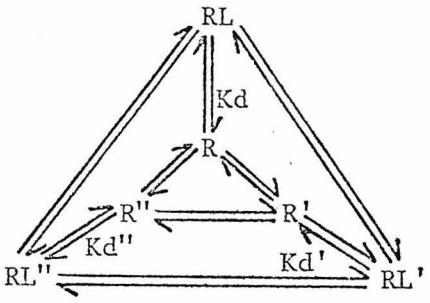
it is an agonist, whereas if the cholinergic ligand binds with high affinity to the R conformation it is an antagonist. This ligand binding scheme is graphically illustrated below:



where a) $N = [R]/[R']$. In the absence of ligand $N >> 1$, and

b) $M = Kd'/Kd$. If $M > 1$ the ligand is an antagonist, if $M < 1$ the ligand is an agonist, and if $M \simeq 1$ the ligand is a depolarizing antagonist.

The two state model proposed by Rang (2) is too simplistic to account for the phenomenon of desensitization. Katz and Thesleff (23) observed that the end plate depolarization in frog sartorius muscle produced by 10 μM carbamylcholine declined after several minutes. They proposed that in the presence of agonist, the receptor changed conformations slowly to a state in which the agonist binds with higher affinity, but the ion translocation device is closed. Furthermore, the potency of antagonists is increased when the end plate region becomes desensitized (24). To account for these observations Lee et al. (25) proposed the three state model shown below:



If we define R, R', Kd, and Kd' as Rang did in the two state model and assume that:

a) R'' designates the desensitized conformation in which the ion translocation device is closed, and

b) both agonists and antagonists bind with highest affinity to the R'' conformation,

this three state model explains adequately the pharmacological data as well as the ligand binding data obtained using membrane preparations enriched for receptor *in vitro*.

4) Biochemical Properties of the Nicotinic Acetylcholine Receptor:

Recent evidence indicates that the affinity of the acetylcholine receptor changes as a function of time after the addition of ligand. Weber *et al.* (26) observed that after the addition of 0.3 μM carbamylcholine, the rate of ^{125}I-α-CoTx binding to *Torpedo marmorata* electroplax membrane preparations enriched for receptor decreased with time. The half time of this process, 3 minutes, far exceeds the time required for the receptor to bind Carb. Furthermore,

if the receptor was first incubated with 0.5 μM Carb, then diluted one hundred fold into Ringer's solution, the receptor bound radioactive α-toxin with the same second order rate constant. Weber et al. (26) proposed that after the addition of cholinergic agonist, the receptor changes conformation slowly to a state which binds cholinergic agonist with 5-20 fold greater affinity.

Lee et al, (25) reinvestigated this phenomenon using membrane preparations of Torpedo californica electroplax enriched for receptor. They observed that the rate of conversion to the high affinity state caused by carbamylcholine was decreased if Ca^{++} was omitted from the Ringers solution, or if the temperature was reduced. These observations are in accord with those of Magazanik and Vyskocil (27,28) who studied the rate of desensitization at the end plate region of frog sartorius muscle. While the precise mechanism of desensitization is not known, these results indicate that the agonist induced effect in vivo and in vitro may be similar.

These time dependent changes in the receptor caused by agonist may affect the ion translocation device. If 2 μM AcCh or 100 μM Carb were added simultaneously to Torpedo marmorata membrane fragments preloaded with $^{22}Na^{+}$ (these microsacs naturally exist as closed vesicles in solution), the relative rate of $^{22}Na^{+}$ efflux from these vesicles was enhanced (29). If the concentrated microsacs were pretreated with sufficient quantities of α-CoTx d-TC, flaxedil, or hexamethonium, this enchancement was blocked.

If the concentrated microsacs were incubated first with 100 μM Carb, then at selected time intervals diluted a hundred fold into 100 μM carbamylcholine, the rate of $^{22}Na^{+}$ efflux declined with time. Sugiyama et al. (30) proposed that after the addition of agonist, the receptor conformation changed with time such that agonist binding no longer leads to increased cation permeability. The half time of this process, which was 2 minutes, decreased if Ca^{++} or the local anesthetic SKF 525-A was included in the incubation medium. Again, this agonist induced effect in vitro may be similar to agonist induced desensitization in vivo.

These data indicate that Torpedo membrane preparations contain both the cholinergic ligand binding site and a functional ion translocation device. Since Eldefrawi et al. (31) postulate that HTx is a specific probe for the ion translocation device and they observed that membrane fragments from Torpedo marmorata electroplax bind both ^{3}H-AcCh and ^{3}H-HTx with high affinity they proposed that these preparations contain both the cholinergic ligand binding site and the ion translocation device. Similar results were obtained by Elliot and Raftery (32). However, it is not known with certainty whether solubilized receptor preparations, which contain the cholinergic ligand binding site, also contain the ion translocation device.

AcCh receptors which contain the cholinergic ligand binding site have been isolated in soluble form from the rat diaphragm (33),

the freshwater eel Electrophorus electricus (34-39) and the elasmobrachs Narcine entemedor (40), Torpedo californica (41,42), Torpedo marmorata (43-44), and Torpedo nobiliana (45). Due to the high density of AcCh receptors per gram wet electroplax, and the local availability of Torpedo californica, we routinely use this as a source of AcCh receptor.

The AcCh receptor isolated from Torpedo electroplax has been characterized in several laboratories. The purified receptor is a glycoprotein (46,47). The molecular weight of the AcCh receptor has been determined using sucrose density gradient centrifugation techniques (48,49), gel filtration techniques (48,50), gel electrophoresis techniques using nondenaturing conditions (47), and membrane osmometry techniques (51), and is estimated to be 300,000 to 400,000 daltons. The receptor is composed of multiple subunits. When subjected to polyacrylamide gel electrophoresis in the presence of SDS and mercaptoethanol, the receptor dissociates into subunits of apparent molecular weights 40,000, 50,000, 60,000, and 64,000 daltons (47,52,53). The molar ratio of these subunits is 4:2:1:1 respectively (47).

The 40,000 subunit may contain a cholinergic ligand binding site. Weill et al. (53) postulated that ^{3}H-4-N-maleimido-α-benzyltrimethylammonium (^{3}H-MBTA) iodide is an affinity reagent for the DTT-reduced receptor. They observed that ^{3}H-MBTA was incorporated preferentially into the 40,000 dalton subunit and

that this incorporation was blocked if the cholinergic ligand binding site was occupied by agonist or antagonist. Similarly, Hucho et al. (54) reacted covalently the photoaffinity label ^{3}H-4-azido-2-nitrobenzyltrimethylammonium fluoroborate with the receptor. All 4 subunits were labelled with this reagent. However, pretreatment of the receptor with α-CoTx blocked incorporation of the label into the 40,000 dalton subunit. The function of the 50,000, 60,000, and 64,000 dalton subunits is not known. The constituent polypeptides of the AcCh receptor contain numerous hydrophobic as well as acidic amino acid residues (47). Thus the amino acid composition of the receptor is consistent with that expected of an integral membrane protein (47,55) as well as with the observed isoelectric point, pI = 4.8, of the purified receptor (43).

CHAPTER I

BINDING OF RADIOACTIVE CHOLINERGIC LIGANDS TO PURIFIED ACETYLCHOLINE RECEPTOR

A procedure for the solubilization and purification of the nicotinic acetylcholine receptor from Torpedo californica electroplax was developed recently (41). The purified receptor has an estimated molecular weight of 380,000 daltons and is composed of multiple subunits (47). While the purified receptor binds ^{125}I-α-BuTx with high affinity, the pharmacological properties of this receptor remain to be determined. We investigated the receptor binding properties using the radioactive ligands acetylcholine, dimethyl-d-tubocurarine, and decamethonium by the technique of equilibrium dialysis.

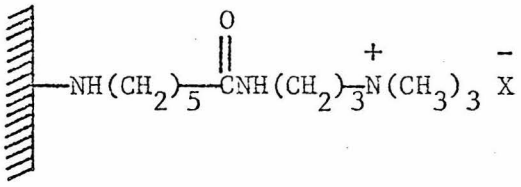
MATERIALS

Diisopropylfluorophosphate was obtained from Aldrich Chemical Co. All radioactive ligands were obtained from Amersham-Searle; their specific activities were: ^{14}C-dimethyl-d-tubocurarine, 83 mCi/mmole, 112 mCi/mmole; ^{14}C-decamethonium, 15 mCi/mmole; ^{3}H-Deca, 400 mCi/mmole; ^{3}H-acetylcholine, 290 mCi/mmole. Carbamylcholine chloride was obtained from Aldrich Chemical Co. and hexamethonium chloride from Mann Laboratories. All other chemicals were reagent grade and obtained from commercial sources. Dialysis tubing was obtained from Union Carbide and was extracted three times with boiling water prior to use. Bungarus multicinctus venom was obtained from Sigma Chemical Co. and ^{125}I-α-BuTx was prepared by the method of Clark et al. (16). Torpedo californica were obtained locally

and the electric organs were excised and used directly, or frozen at -90°C.

METHODS

Preparation of Receptor. Solubilized AcCh receptor was obtained using the membrane fractionation and extraction procedure outlined in figure 2. The solubilized receptor was purified using the affinity chromatography techniques as described by Schmidt and Raftery (41). In brief, the receptor solution was adsorbed onto a column containing a Sepharose 2B resin to which the following ligand is attached covalently:

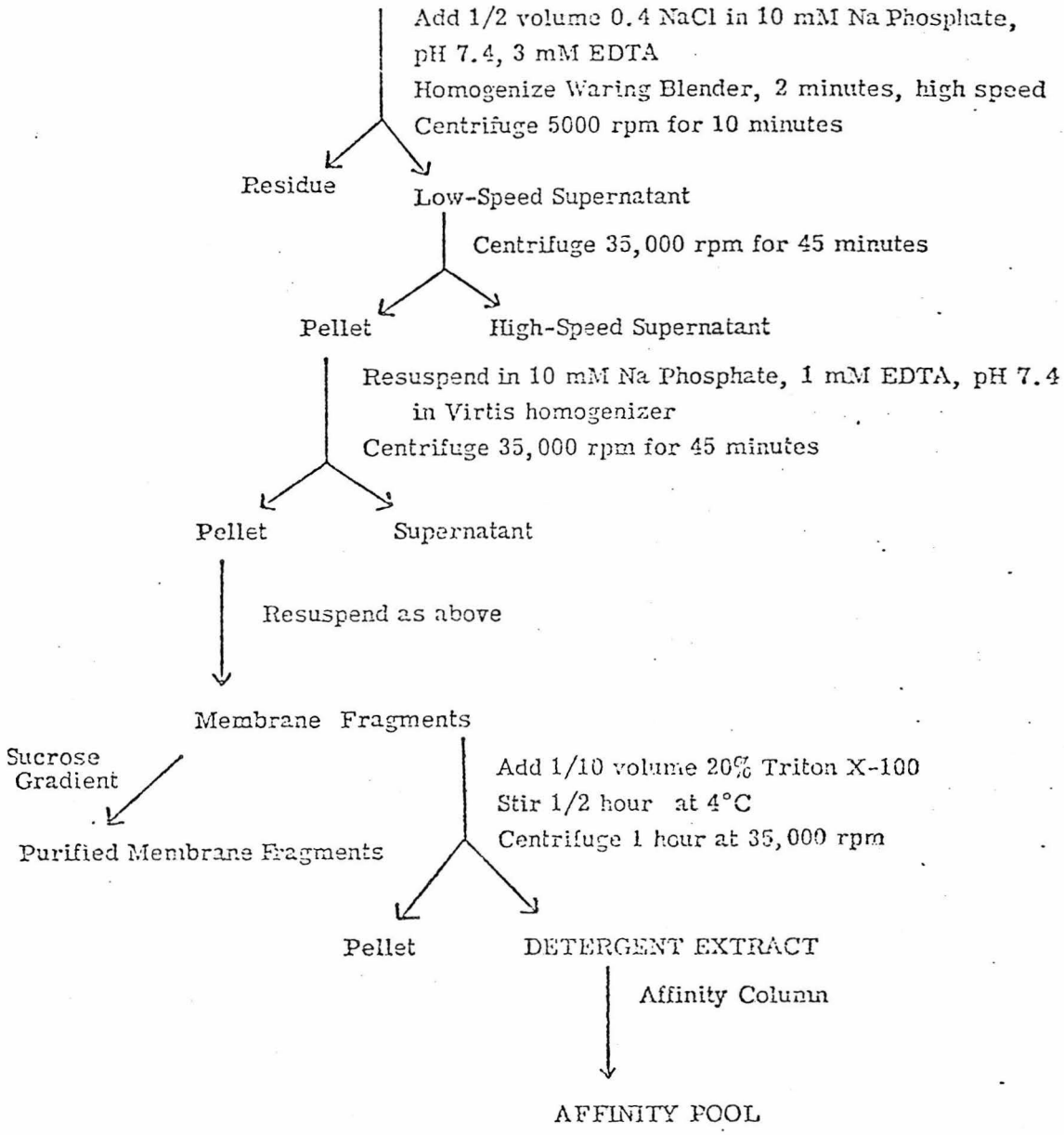


A NaCl gradient from 0 to 1 M was applied and the receptor eluted when [NaCl] = 70 mM. The fractions enriched for ^{125}I-α-BuTx binding were pooled and concentrated using an Amicon ultrafiltration cell with a PM-30 filter. For use in AcCh binding studies, the purified receptor solution was treated with 100 μM DFP to inactivate residual AcCh esterase. The DFP treatment did not affect ^{125}I-α-BuTx, ^{3}H-Deca, or ^{14}C-dimethyl-dTC binding to the receptor. The treated preparation was dialyzed extensively against 0.1% Triton X-100 and 10 mM Na phosphate (pH 7.4) and filtered through a 0.45 micron

Figure 2.

Membrane fractionation and extraction procedure used for isolation of the AcCh receptor from Torpedo californica electroplax.

ELECTRIC ORGAN



HAWP Millipore filter to remove particulate matter. The final solution had a protein concentration of 0.5-2 mg/ml as determined by the method of Lowry et al. (56) using BSA as standard. The receptor preparation normally bound 10 nmoles ^{125}I-α-BuTx per mg of protein.

Equilibrium Dialysis. Two hundred microliters of the AcCh receptor solution were placed in 1 cm dialysis tubing. The bag was placed in a 10 ml screw cap vial containing 5 ml of 10 mM Na phosphate, 200 mM MaCl, and 0.1% Triton X-100 (pH 7.3). The buffer contained high concentrations of NaCl to minimize Donnan equilibrium effects (57). The appropriate concentration of radioactive ligand was added and the vial placed on a horizontal rocker (Buchler Instruments) and agitated gently for 16-20 hours at 4°C. The radioactive ligands equilibrated fully in this time period. During the dialysis procedure the protein concentration remained constant as did the ^{125}I-α-BuTx binding activity. This indicates that the receptor was neither diluted nor inactivated during the dialysis procedure.

Duplicate 0.050 ml aliquots of the dialysis bag contents and the dialysate were removed, solubilized in 0.5 ml NCS tissue solubilizer (Amersham), and counted in 8 ml of 0.4% Permablend TM III (Packard) in toluene. The amount of free ligand was calculated from the radioactivity in the external medium, whereas the amount of bound ligand was calculated on the basis of the

radioactivity in the dialysis bag contents minus the radioactivity in the external medium. The amount of AcCh hydrolyzed was determined using the method of Johnson and Russel (58). Normally less than 10% of the ^{3}H-AcCh was hydrolyzed during dialysis. The hydrolysis product ^{3}H-acetate did not bind to the receptor with detectable affinity, nor did the released choline appear to inhibit the binding of ^{3}H-AcCh.

Competition experiments. The binding experiments were conducted as described above except that a given concentration of cholinergic ligand was added to the dialysate. Hexamethonium, d-tubocurarine, and carbamylcholine were used as inhibitors. The data were analyzed using the ligand binding scheme shown below:

$$R + L \underset{Kd}{\rightleftharpoons} RL \qquad Kd = [R][L]/[RL]$$

$$+ \qquad\qquad\qquad Ki = [R][I]/[RI]$$

$$I \;\Big\|\uparrow\downarrow\; Ki$$

$$RI$$

$$[Ro]/[R] = ([RL] + [RI] + [R])/[RL]$$

$$= 1 + ([RI] + [R])/[RL]$$

$$= 1 + Kd/[L](1 + [I]/Ki)$$

$$1/[RL] = 1/[Ro] + 1/[Ro](Kd/[L])(1 + [I]/Ki) \qquad (1)$$

where [L] is the free radioactive ligand concentration, [Ro] is the total concentration of radioactive ligand binding sites, and [I]

is the concentration of free cholinergic ligand.

α-BuTx Binding Assay. The total concentration of ^{125}I-α-BuTx binding sites was determined using the method of Schmidt and Raftery (59). Excess ^{125}I-α-BuTx was added to the AcCh receptor solution (buffer is 10 mM Na phosphate and 0.1% Triton X-100 (pH 7.4)). After 30 minutes, 100 µl aliquots are pipeted onto a Whatman DE-81-DEAE cellulose disc. Since the receptor-^{125}I-α-BuTx complex has an apparent pI of 5.15, whereas free ^{125}I-α-BuTx has an apparent pI of 9.5, the receptor-toxin complex adsorbs preferentially to the DEAE filter disc (48). Most of the free ^{125}I-α-BuTx is removed from the disc by three consecutive washes in 10 mM sodium phosphate, 50 mM NaCl, and 0.1% Triton X-100 for 10 minutes. The discs are removed, partially dried on paper towels, and counted in 10 ml of 0.55% Permablend III in Triton/toluene (1:3,v:v) (60). Since the receptor binds the toxin in an essentially irreversible manner (17), this assay represents a rapid and quantitative method of estimating the total concentration of cholinergic ligand binding sites.

RESULTS

Figure 3 shows a double reciprocal plot of ^{14}C-dimethyl-d-TC binding to the purified receptor. The data were fit by a straight line indicating that ^{14}C-dimethyl-d-TC binds to a single class of noninteracting sites. Figure 4 is a Hill plot of the binding data

obtained using ^{3}H-AcCh. The slope of the line, which is approximately unity, indicates that ^{3}H-AcCh appears to bind to a single class of noninteracting sites. Similar data were obtained using radioactive Deca and dimethyl-d-TC. The binding data for all ligands used are summarized in Table I. Under the experimental conditions used here, the receptor binds AcCh, dimethyl-d-TC, and Deca with decreasing affinities respectively. Each of the ligands appears to bind with high affinity to approximately 50% as many sites as ^{125}I-α-BuTx. Since pretreatment of the receptor with a two fold excess of α-BuTx blocks all detectable ligand binding, these radioactive ligand binding sites may be physiologically important in vivo.

Competition experiments were conducted using hexamethonium, d-tubocurarine, and carbamylcholine as inhibitors. Figure 5 illustrates that carbamylcholine competitively inhibits ^{3}H-AcCh binding. Also we observed that hexamethonium competitively inhibits ^{3}H-Deca binding. However, carbamylcholine does not competitively inhibit ^{14}C-dimethyl-d-TC or ^{3}H-Deca binding nor does hexamethonium competitively inhibit ^{14}C-dimethyl-d-TC or ^{3}H-AcCh binding. Similarly, d-tubocurarine does not competitively inhibit ^{3}H-AcCh or ^{3}H-Deca binding. These results indicate that the cholinergic ligands tested do not bind to a homogeneous class of sites.

Figure 3.

Double reciprocal plot illustrating the binding of ^{14}C-dimethyl-d-tubocurarine to the purified receptor. The buffer contained 10 mM Na phosphate, 200 mM NaCl, and 0.1% Triton X-100 (pH 7.3).

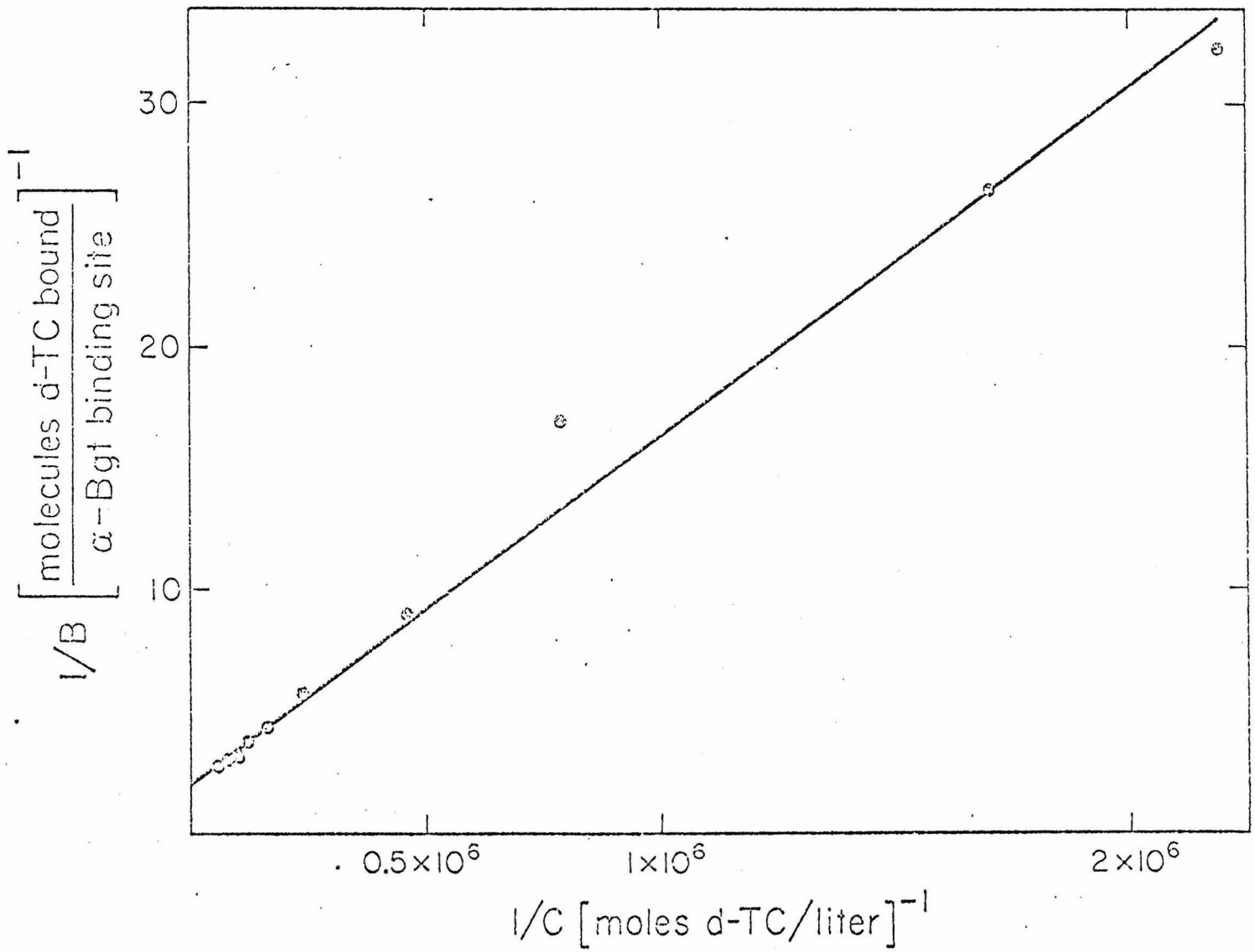

30
20
10
0.5×10^6
1×10^6
2×10^6
1/B [molecules d-TC bound / α-Bgt binding site]^-1
1/C [moles d-TC/liter]^-1

Figure 4.

Hill plot illustrating the binding of ^{3}H-acetylcholine to the purified receptor. The buffer was composed of 10 mM Na phosphate, 200 mM NaCl, and 0.1% Triton X-100 (pH 6.4). The slope of the line, n, = 0.94.

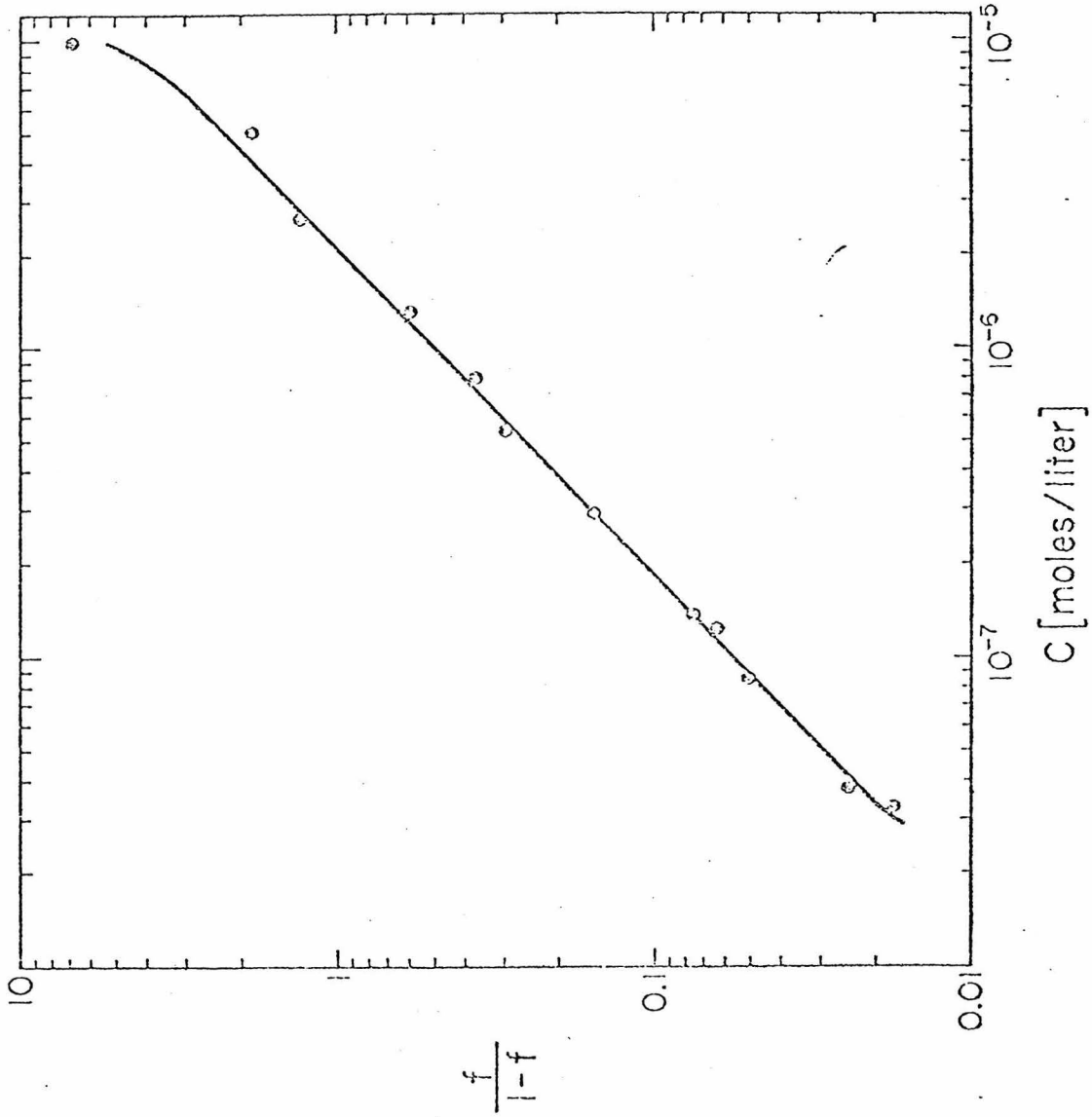

10
1
0.1
0.01
$\frac{f}{1-f}$
10^{-5}
10^{-6}
10^{-7}
C [moles/liter]

TABLE I

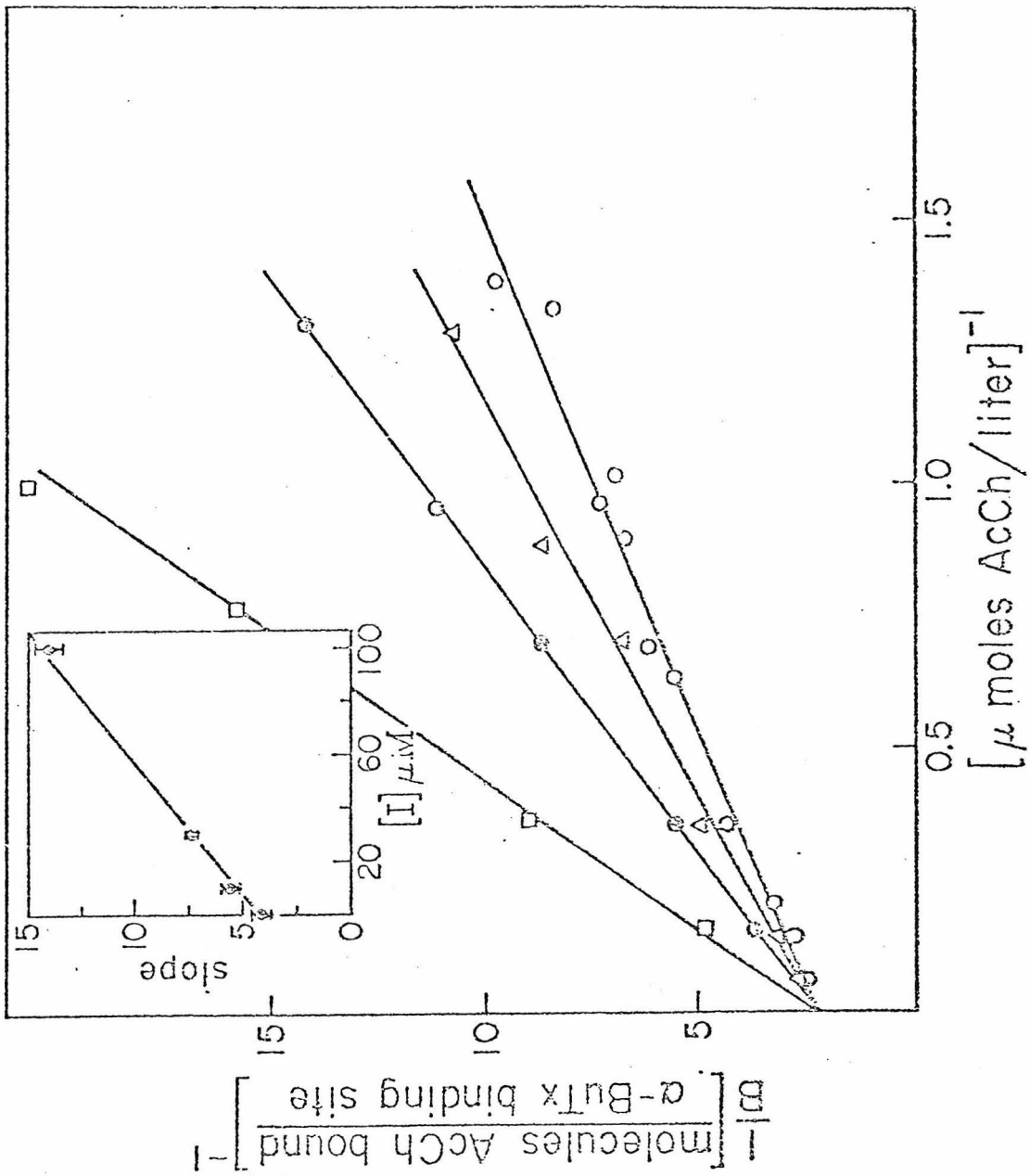
Summary of radioactive ligand binding data to purified acetylcholine receptor.

Ligand	Kd	B max/B(α-BuTx)	n (Hill coefficient)
AcCh	2.3 ± .3 μM	.42 ± .03	.93 ± .03
dimethyl-d-TC	6.4 ± .5 μM	.52 ± .05	1.05 ± .10
Deca	55 ± 15 μM	.41 ± .15	1.08 ± .03

Binding data were obtained at 4°C. using the technique of equilibrium dialysis. Buffer contained 10 mM Na phosphate, 200 mM NaCl, and 0.1% Triton X-100 (pH 7.3).

Figure 5.

Double reciprocal plot of ^{3}H-acetylcholine binding to the purified receptor in the absence of (○), and presence of 10 μM (△), 30 μM (◍), and 100 μM (□) carbamylcholine. The buffer contained 10 mM Na phosphate, 200 mM NaCl, and 0.1% Triton X-100 (pH 7.3).
Insert: Replot of the apparent slope versus the carbamylcholine concentration. The calculated Ki for carbamylcholine = 40 μM.


$\frac{1}{B}$ [molecules AcCh bound / α-BuTx binding site]$^{-1}$
15
10
5
0.5
1.0
1.5
[μ moles AcCh/liter]$^{-1}$
slope
15
10
5
0
20
60
100
[I] μM

DISCUSSION

Since the ligand binding properties of the purified AcCh receptor from Torpedo californica electroplax have not been investigated, we studied the receptor-ligand interactions using the technique of equilibrium dialysis. The receptor appears to bind radioactive AcCh, dimethyl-d-TC, and Deca with decreasing affinities respectively to half as many sites as ^{125}I-α-BuTx. Each ligand appears to bind to a single class of noninteracting sites.

These sites, however, do not appear to be homogeneous. While carbamylcholine inhibits ^{3}H-AcCh binding competitively, it does not inhibit ^{3}H-Deca or ^{14}C-dimethyl-d-TC binding competitively. Similarly, hexamethonium inhibits ^{3}H-Deca binding competitively, but does not inhibit ^{14}C-dimethyl-d-TC or ^{3}AcCh binding competitively. Also d-tubocurarine does not inhibit ^{3}H-AcCh or ^{3}H-Deca binding competitively. The simplest interpretation of these data is that carbamylcholine and acetylcholine bind to the same site as do hexamethonium and decamethonium. However, acetylcholine, decamethonium, and dimethyl-d-tubocuraine are not mutually exclusive.

Since the observed inhibition data that were not competitive could not be fit assuming any simple binding scheme, we postulate that the radioactive cholinergic ligands do not bind to a single class of sites, and/or the site which binds these ligands with high

affinity is composed of several subsites such that the decamethonium, acetylcholine, and dimethyl-d-tubocurarine binding sites only partially overlap. Recently, Raftery et al. (61,62) proposed that the purified receptor contains two classes of α-BuTx binding sites in equal populations. They observed that the "fast" site binds α-BuTx with a five fold greater second-order rate constant and cholinergic ligands with 2-7 fold lower affinity than does the "slow" site. While the experimental conditions used by Raftery et al. differ from those used here, we propose that that the radioactive cholinergic ligands used here bind predominantly to the "slow" site. In Chapter II, we show that the "slow" site is composed of at least two subsites.

CHAPTER II

BINDING OF SYNTHETIC FLUORESCENT PROBES TO PURIFIED ACETYLCHOLINE RECEPTOR

Recently, Raftery et al. (61,62) and Reed et al. (63) studied the rate of radioactive α-BuTx binding to the solubilized and purified AcCh receptor in the presence and absence of various cholinergic ligands. They observed that in 10 mM Na phosphate, 100 mM NaCl, and 0.1% Triton X-100 (pH 7.25 at 25 °C.), α-BuTx bound to two equal populations of receptor sites with significantly different second order rate constants, $k_{2I} = 1.3 \times 10^6\ M^{-1}sec^{-1}$ and $k_{2II} = 1.9 \times 10^5\ M^{-1}sec^{-1}$. In the presence of cholinergic ligands the rate of toxing binding to the "slow" site (characterized by k_{2II}) was decreased to a greater degree than was the rate of toxin binding to the "fast" site. Assuming that these ligands are competitive inhibitors of toxin binding, they proposed that cholinergic ligands bind with slightly higher affinity to the "slow" than the "fast" toxin binding site; the values calculated for carbamylcholine binding are Kd_{II} = 60 μM (for the "slow" site) and Kd_I = 170 μM (for the "fast" site), and for decamethonium binding Kd_{II} = 12 μM and Kd_I = 80 μM. However, they observed that the fluorescent analogue of decamethonium, bis-3-aminopyridinium-1,10-decane (DAP) bound with substantially greater affinity to the "slow" than the "fast" site (Kd_{II} = 0.3 μM and Kd_I = 45 μM). These latter results are consistent with those of Martinez-Carrion and Raftery (64) in which DAP bound tightly to only half as many sites as α-BuTx. Since DAP binds almost exclusively to the "slow" toxin binding site, we investigated receptor-ligand interactions

at this site using a series of synthetic bis-3-aminopyridinium-1,n-alkane fluorescent probes.

MATERIALS

3-Aminopyridine, 1,6-diiodohexane, 1,8-diiodooctane, 1,10-diiododecane, and 1,12-diiododododecane were obtained from K and K Laboratories. 1,14-dibromotetradecane, 1,16-dibromohexadecane, and 1,18-dibromooctadecane, and decamethonium dibromide were obtained from ICN Pharmaceuticals. 1,4-diiodobutane and carbamylcholine chloride were obtained from Sigma Chemical Co. 1-Iodopropane and iodomethane were obtained from Matheson, Coleman, and Bell. Lyophilized Bungarus multicinctus venom was obtained from Sigma Chemical Co. and ^{125}I-α-BuTx was prepared using the method of Clark et al. (16). Torpedo californica were obtained locally and the electric organs were excised and used directly or frozen at -90°C.

METHODS

Fluorescent Probe Synthesis. Fluorescent probes were synthesized using the method of Mooser et al. (65). The bifunctional probes were synthesized by refluxing approximately 4 g of the dihaloalkane with 8 g of 3-aminopyridine for 85 hours in 60 ml of acetone. The monofunctional probes were synthesized by refluxing equimolar amounts of iodoalkane and 3-aminopyridine for 18 hours in 60 ml of acetone. The acetone solutions were

reduced to near dryness on a rotary evaporator. The dark colored precipitates which remained were redissolved in absolute ethanol, treated with decolorizing carbon, and recrystallized 3 to 5 times from ethanol. Off-white crystals were recovered in a 20-50% yield. An elemental analysis and melting point determination were performed of each final product. Table II shows that the observed and theoretical elemental analyses are in good agreement. Thin layer chromatography analyses were performed using cellulose plates obtained from Kodak. The solvent contained butanol/ethanol/water/acetic acid (4:2:1:1,v:v:v:v) and each product ran as a single spot. Proton nuclear magnetic resonance analyses yielded spectra which were consistent with those predicted for each product.

Purification of the AcCh Receptor. Receptor was purified from T. californica electroplax using affinity chromatography techniques as described by Schmidt and Raftery (40,41). Proteolysis of subunits was minimized by taking the precautions outlined by Raftery et al. (50,61,62) and Vandlen and Raftery (47). The solubilized receptor solution was concentrated using an Amicon ultrafiltration cell with a PM-30 membrane. The concentrated protein solution was dialyzed at 4°C. against buffer containing 10 mM Na phosphate and 0.03% Triton X-100 (pH 7.4) for use in binding studies under conditions of low ionic strength (Buffer A) or 10 mM Na phosphate, 0.03% Triton X-100, and 100 mM NaCl (pH 7.25) for use in binding studies under conditions of high ionic strength

TABLE II. Analysis of the Synthetic 3-Aminopyridinium Salts.

Probe	%C found	%C theoretical	%N found	%N theoretical	%H found	%H theoretical	mp(°C)
3-Aminopyridinium-(1-methyl) iodide	30.69	30.52	11.80	11.87	3.87	3.84	119.5 - 121.5
3-Aminopyridinium-(1-propyl) iodide	36.42	36.38	10.57	10.61	4.97	4.96	119 - 120
BAP diiodide	33.80	33.76	11.21	11.25	4.21	4.05	231 - 233
HAP diiodide	36.63	36.52	10.58	10.65	4.53	4.60	253.5 - 255.5
OAP diiodide	39.12	39.01	9.97	10.11	5.14	5.09	181.5 - 183
DAP diiodide	41.40	41.25	9.46	9.62	5.41	5.54	178.5 - 181
DoDAP diiodide	43.45	43.29	9.12	9.18	5.89	5.95	175 - 176.5
TetraDAP dibromide	51.09	52.95	9.89	10.29	7.46	7.41	161.5 - 163
HexaDAP dibromide	53.89	54.55	9.48	9.79	7.78	7.75	159 - 161.5
OctaDAP dibromide	55.64	56.00	9.17	9.33	8.04	8.06	163 - 164.5

(Buffer B). The solubilized receptor solution was then filtered through a 0.45 micron HAWP Millipore filter to remove particulate matter and stored at 4°C. until use. Since Triton X-100 (polyoxyethylene-$_p$-t-octylphenol) contributed to the relative fluorescence intensity background, the Triton X-100 concentration was reduced in the buffers from 0.1 to 0.03%. Using this detergent concentration the receptor remained in solution and no difference in the receptor ligand or toxin binding properties was observed (we calculate that this detergent concentration is approximately two fold greater than the critical micelle concentration (66)).

Characterization of the Purified Acetylcholine Receptor. DEAE cellulose disc assays were performed as described by Schmidt and Raftery (59) to determine the total concentration of ^{125}I-α-BuTx binding sites at equilibrium. Protein concentration was determined by the method of Lowry et al. (56) using BSA as standard. Routinely, the purified receptor bound 5-10 nmoles α-BuTx per mg protein. The integrity of the purified receptor was assessed by SDS polyacrylamide gel electrophoresis in the presence of 1% mercaptoethanol using the procedure of Laemmli (67). The stacking and separating portions of the gels contained 3 and 12.5% polyacrylamide at an acrylamide to bis ratio of 125:1 (w:w). Routinely, Coomassie blue stained bands of apparent MW 40,000, 50,000, 60,000, and 64,000 daltons were observed indicating that little or no proteolysis had occurred (47,50,52,61,62,68).

Fluorescent Ligand Titrations. The fluorescence intensity background of solutions containing:

a) receptor (0.2-8 μM in ^{125}I-α-BuTx binding sites),

b) receptor pretreated with excess α-BuTx, and

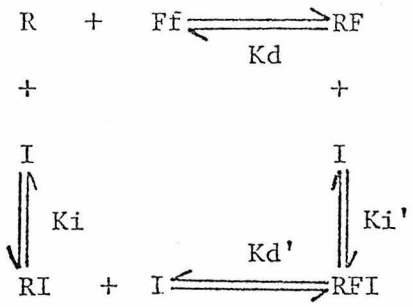
c) buffer only,

was determined at 25°C. on a Perkin Elmer MPF-4 spectrofluorimeter equipped with a thermostated chamber. A given concentration of fluorescent probe was then added to each solution. The maximum excitation and emission wavelengths of the fluorescent probes were 330 and 405 nm respectively. The synthetic probes were highly fluorescent in aqueous solution but the fluorescence was quenched by greater than 95% when bound to the receptor. Other investigators have observed that the fluorescence of DAP is almost totally quenched when bound to AcCh esterase from Electrophorus electricus (69) and Torpedo californica (70) or AcCh receptor from Torpedo californica (64). Therefore, the relative fluorescence intensity in the presence of probe minus the relative fluorescence intensity in the absence of probe for solution a) represents the concentration of free probe [Ff]. The relative fluorescence intensity for solution b) represents [Ff] + [Fb], where [Fb] is the concentration of probe quenched specifically. The fluorescence intensity difference for solution c) represents [Fo], where [Fo] is the total probe concentration.

Direct Binding Data. Routinely, increasing amounts of

concentrated probe were added until [Fb] appeared to be maximal. In general, less than 75 microliters total of concentrated probe was added to the 2.5 ml reaction mixture. If [Fb]/[Ff] was plotted against [Fb] as a function of different probe concentrations, a straight line was obtained, indicating that the probes bound to a single class of noninteracting sites. The Kd and total concentration of probe sites, [Ro], were calculated as described by Scatchard (71). Binding of a series of synthetic mono- and bifunctional probes was studied in this manner. The titrations were performed using both Buffers A and B.

Fluorescent Probe Displacement Studies. Routinely, sufficient quantities of probe were added so that 50% of the available probe sites were occupied ([Ff] = Kd), and the concentration of free probe was determined. Then concentrated cholinergic ligand was added and the [Ff] redetermined. This process was repeated until [Ff] was maximal. Usually, less than 100 microliters total of concentrated cholinergic ligand were added to the 2.5 ml reaction mixture. The effects of carbamylcholine and decamethonium upon bifunctional probe binding were determined using both Buffers A and B. The data was analyzed using the ligand binding scheme shown below:



$$Kd = [R][Ff]/[RF]$$

$$Kd' = [RI][Ff]/[RFI]$$

$$Ki = [R][I]/[RI]$$

$$Ki' = [RF][I]/[RFI]$$

$$[Ro]/([RF] + [RFI]) = 1 + ([R] + [RI])/([RF] + [RFI])$$

$$" = 1 + Kd/[Ff](1 + [I]/Ki)/(1 + [I]/Ki')$$

$$[Ro](1 + [I]/Ki') = ([Fo] - [Ff])(1 + [I]/Ki) + ([Fo] - [Ff])Kd/[Ff](1 + [I]/Ki)$$

$$[Ff] = \frac{-[([Ro] - [Fo])(1 + [I]/Ki') + Kd(1 + [I]/Ki)] + \{[([Ro] - [Fo])(1 + [I]/Ki') + Kd(1 + [I]/Ki)]^2 + 4[Fo]Kd(1 + [I]/Ki)(1 + [I]/Ki')\}^{1/2}}{2(1 + [I]/Ki')}$$

where [Ro], [Ff], and [Fo] were defined previously, and [I] if the concentration of free cholinergic ligand (since the total concentration of cholinergic ligand added is significantly greater that [Ro], the total approximates the free). The data were fit using a nonlinear least squares regression analysis. Since [Fb] = [Fo] - [Ff], the concentration of bound probe was calculated from the observed [Ff], and the data plotted in terms of percentage of bound probe released as a function of log [I].

Toxin Binding Kinetics. The rate of ^{125}I-α-BuTx binding to the receptor was determined in the presence and absence of varying concentrations of fluorescent probe by the method of Raftery et al.

(61,62) and Reed et al. (63) using Buffer B. Solutions containing AcCh receptor were incubated for 10 minutes with fluorescent probe, then excess ^{125}I-α-BuTx was added and the solution quickly mixed. At given time intervals, 100 µl aliquots were withdrawn and the amount of ^{125}I-α-BuTx-receptor complex determined. The data were fit to equation 3 using a nonlinear least squares regression analysis:

$$R_I + To \xrightarrow{k_{2I}} R_I T$$

$$R_{II} + To \xrightarrow{k_{2II}} R_{II} T$$

$$2C_t = C_\infty(2 - e^{-k_I t} - e^{-k_{II} t})$$

$$\ln[2(C_\infty - C_t)/C_\infty] = \ln(e^{-k_I t} + e^{-k_{II} t}) \qquad (3)$$

where C_t represents the concentration of bound ^{125}I-α-BuTx at time t, $2C_\infty$ is the concentration of bound toxin when the reaction is complete, and k_I and k_{II} are the observed pseudo first order rate constants for toxin binding to the "fast" and "slow" site respectively. Kd_I and Kd_{II}, which are the probe concentrations required to decrease the rate of α-BuTx binding by 50% to the "fast" and "slow" sites respectively, were calculated by performing a linear least squares regression analyses to the following equations:

$$k_I = k_{2I} To(1 + L/Kd_I)$$

$$k_{II} = k_{2II} To(1 + L/Kd_{II}) \qquad (4)$$

where To is the total toxin concentration, L is the concentration of fluorescent probe added (since L is significantly greater than

the concentration of α-BuTx binding sites, the total concentration of probe added approximates the free), and k_{2I} and k_{2II} are the calculated second order rate constants of toxin binding to the "fast" and "slow" site respectively in the absence of fluorescent probe. If the probes equilibrate rapidly with the receptor relative to the toxin, and the probes competitively inhibit toxin binding the calculated Kd_I and Kd_{II} values represent equilibrium dissociation constants for probe binding to the "fast" and "slow" site respectively.

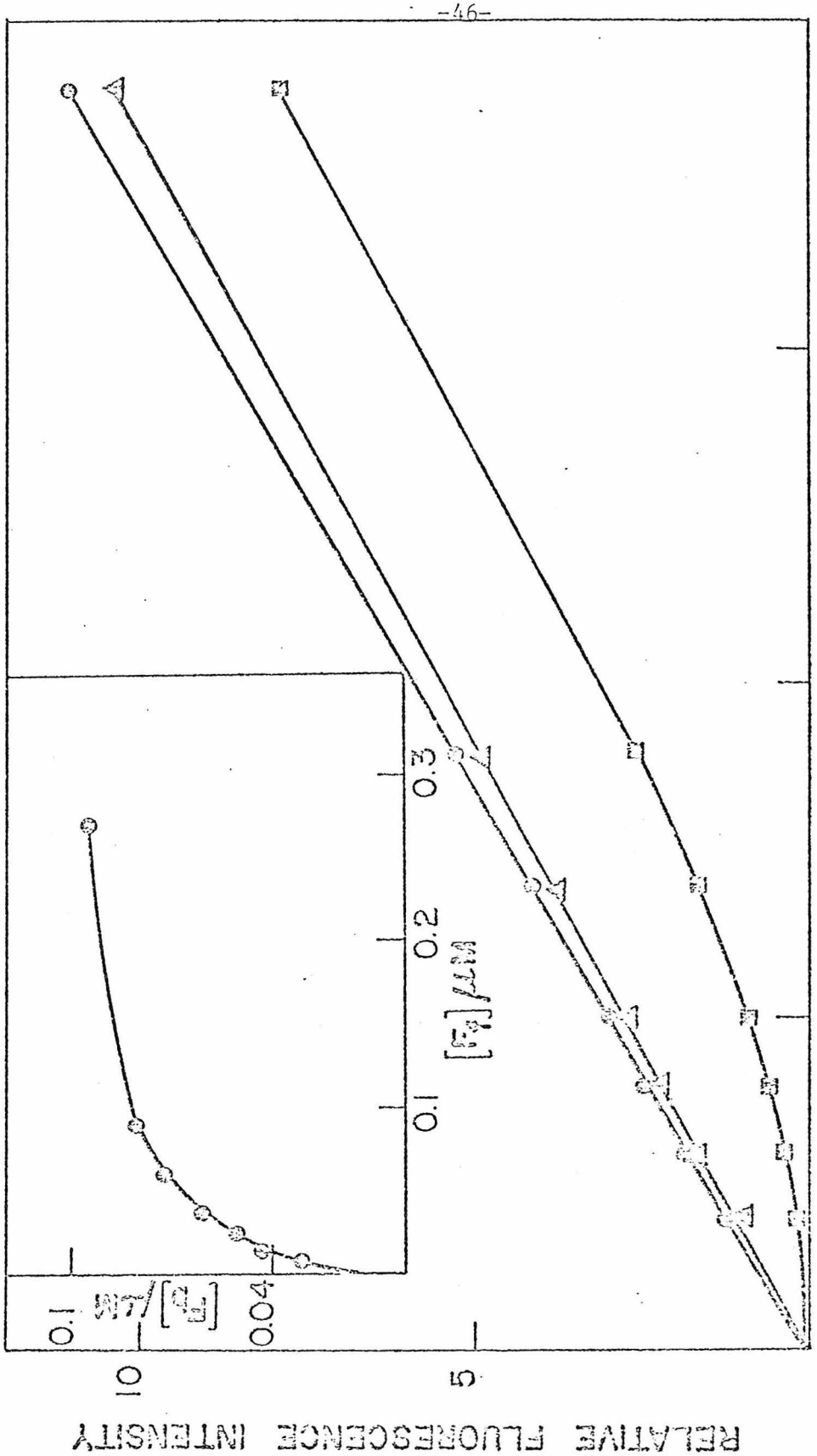
RESULTS

Direct Binding Studies. Figure 6 shows the relative fluorescence intensity of DoDAP versus total probe concentration a) in the presence of receptor, b) with receptor pretreated with a two fold excess of α-BuTx, and c) in the absence of receptor. The fluorescence intensity versus total probe concentration in the receptor free and the α-BuTx treated receptor solutions was linear, but a measurable quantity of fluorescent probe was quenched in the latter. This quenching may result from probe binding with low affinity to sites not blocked by α-neurotoxin. The fluorescence intensity versus total probe concentration in the receptor solution increased slowly with a hyperbolic lag, then increased linearly with the same apparent slope as solution b). This indicates that at low probe concentrations, the bulk of the total probe

Figure 6.

Plot of the relative fluorescence intensity of DoDAP (corrected for background) as a function of the total amount of probe added to buffer only (●), receptor pretreated with 0.4 M α-BuTx (▲), and receptor (■). The purified receptor concentration was 0.2 μM in ^{125}I-α-BuTx binding sites. Buffer A was used.

Insert: Plot illustrating the calculated concentration of quenched probe versus the concentration of free probe.


RELATIVE FLUORESCENCE INTENSITY
10
5
0.1
0.04
1/[F] μM⁻¹
0.1
0.2
0.3
[F] μM
0.10
0.20
0.30
TOTAL [D₀DAP] μM

added was quenched, whereas at high probe concentrations the high affinity DoDAP binding sites were saturated. Figure 6 insert shows that a replot of the amount of DoDAP bound versus free appears to be hyperbolic.

Figure 7 is a Scatchard replot of a similar experiment which was conducted using OAP. The data were fit by a straight line indicating that OAP bound to a single class of noninteracting sites. The Scatchard plots obtained using the other bifunctional probes were similar in form to that shown here.

Figure 8 summarizes the binding data obtained using the bifunctional probes in Buffer A. The receptor binds the bifunctional probes with increasing affinity as the length of the methylene bridge (n) between the two 3-aminopyridinium rings is increased from 4 to 12 and decreased from 18 to 16. The receptor bound DoDAP (n = 12), TetraDAP (n = 14), and HexaDAP (n = 16) with similar affinities. Table III shows that the receptor did not bind the synthetic monofunctional probes with appreciable affinity. The simplest interpretation of these data is that the receptor can interact strongly with the bifunctional probes at two sites. The receptor binds DoDAP, TetraDAP, and HexaDAP with highest affinity because these ligands are of the proper conformation so that they interact maximally with each of the receptor subsites. Bifunctional probes in which n <10 or monofunctional probes interact maximally with only one of the receptor subsites and thus bind

Figure 7.

Scatchard plot of OAP binding in Buffer A (●) and Buffer B (○) when the purified receptor concentration was 0.6 μM in ^{125}I-α-BuTx binding sites. The solid lines were calculated from a linear least squares regression analysis of the data. The calculated Kd is 0.26 ± 0.03 μM using Buffer A (●) and 0.95 ± 0.12 μM using Buffer B (○).

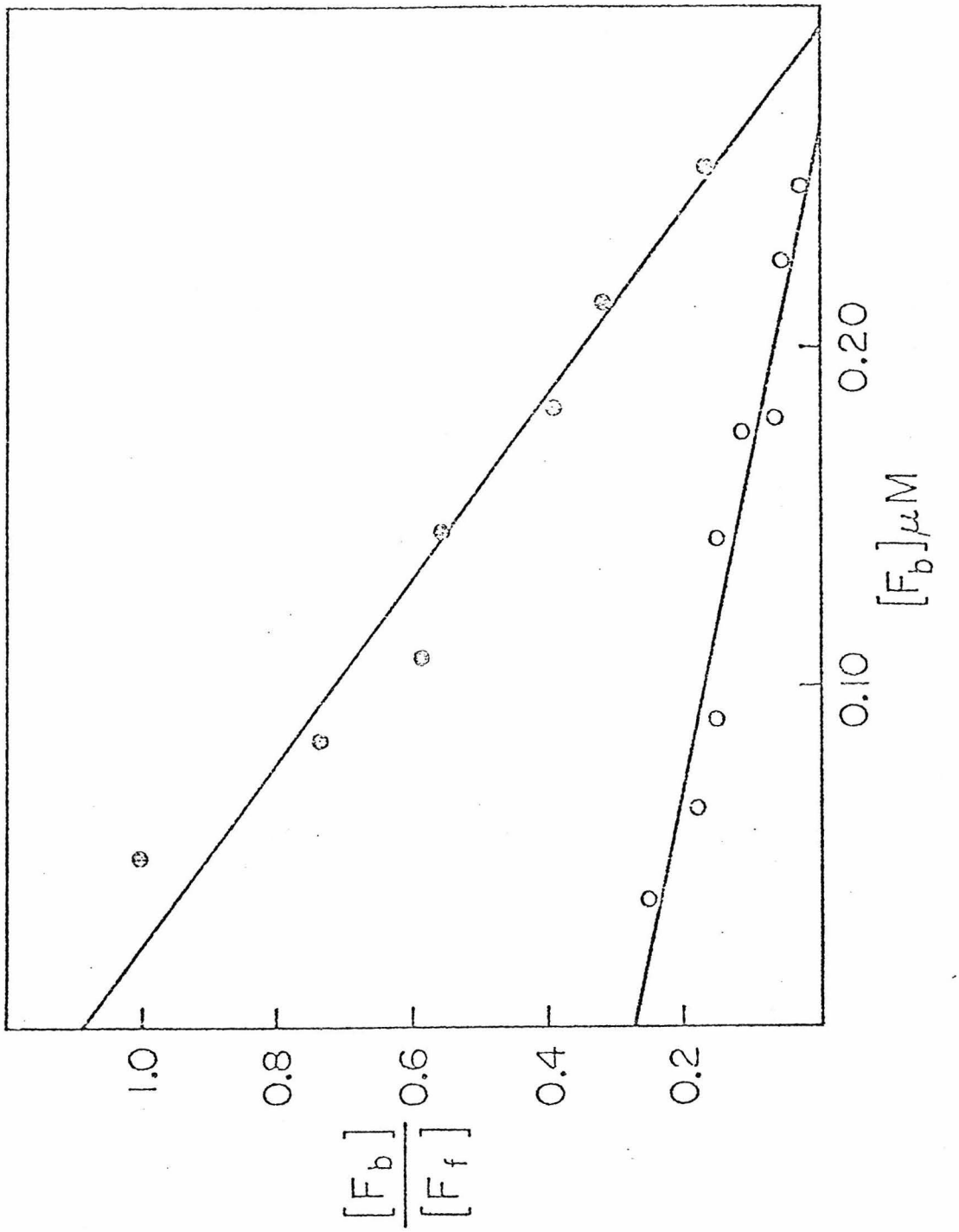

1.0
0.8
0.6
0.4
0.2
[Fb]/[Ff]
0.10
0.20
[Fb]μM

Figure 8.

Plot illustrating the log Kd for the synthetic bis-3-aminopyridinium-1,n-alkane fluorescent probes as a function of the length of the methylene bridge (n). Buffer A is used.

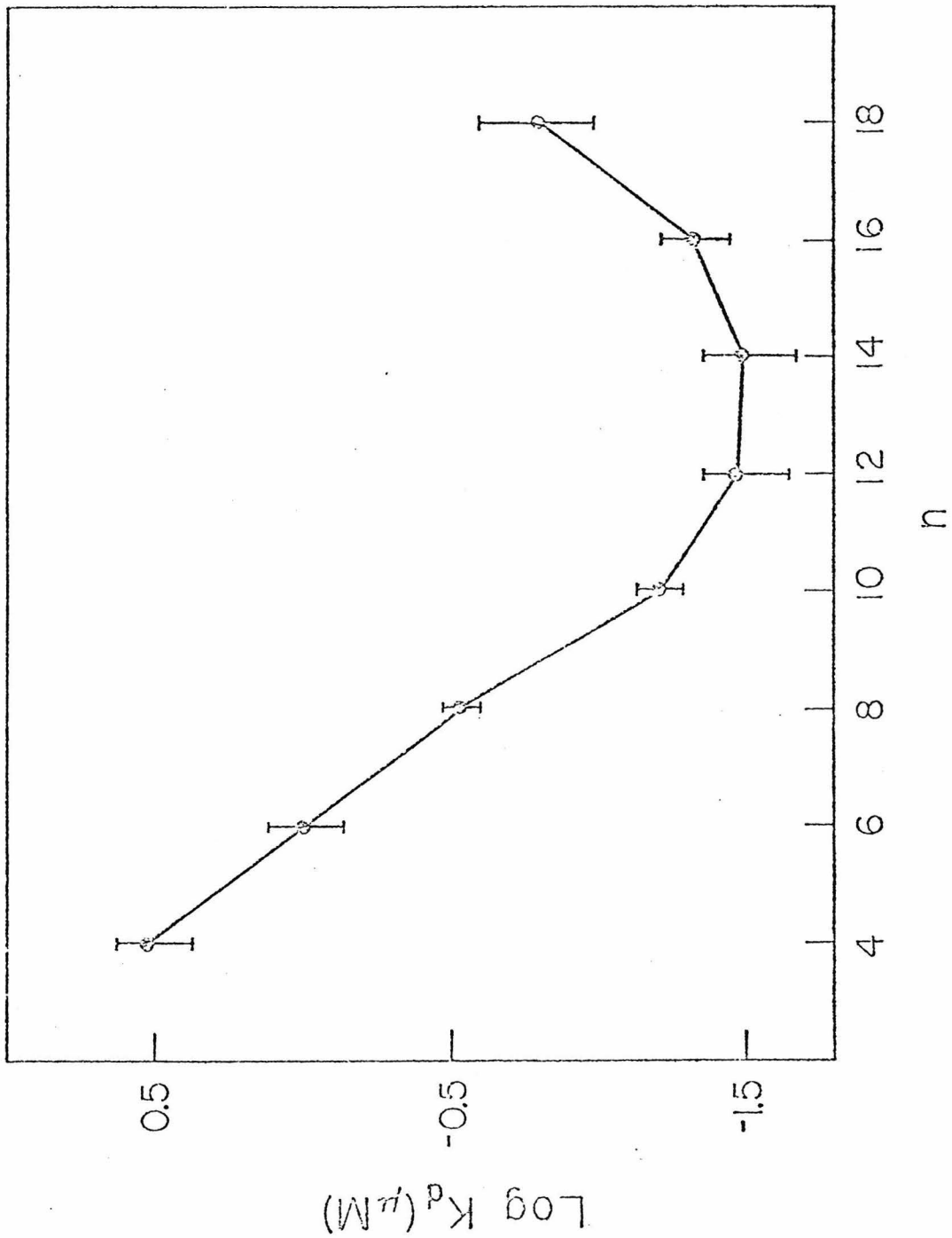

Log K_d (μM)
0.5
-0.5
-1.5
4
6
8
10
12
14
16
18
n

TABLE III
LIGAND BINDING PARAMETERS

PROBE	K_d (μM) [a] BUFFER A IS USED	K_d (μM) [a] BUFFER B IS USED	BUFFER B IS USED $K_{d_{II}}$ [b]	K_{d_I} [b]
OctaDAP	0.175 ± 0.075 (4)	0.67 (2)	n.d.	n.d.
HexaDAP	0.047 ± 0.012 (5)	0.15 (1)	n.d.	n.d.
TetraDAP	0.032 ± 0.011 (5)	0.11 (1)	n.d.	n.d.
DoDAP	0.034 ± 0.010 (7)	0.19 (2)	0.32 ± 0.03	> 10
DAP	0.061 ± 0.011 (7)	0.42 ± 0.09 (4)	0.71 ± 0.04	> 10
OAP	0.295 ± 0.045 (5)	0.95 (2)	0.64 ± 0.12	> 5
HAP	1.03 ± 0.29 (5)	3.7 (3)	4.2 ± 0.5	> 40
BAP	3.35 ± 1.0 (5)	> 10	24 ± 7	80 ± 30
3-Aminopyridinium-(1-methane)	> 10	n.d.	1500 ± 300	2000 ± 300
3-Aminopyridinium-(1-propane)	> 10	n.d.	260 ± 60	560 ± 120

n.d. represents not determined. [a]The experiments were performed as described in Methods using the fluorimetric assay. The K_d values expressed represent a mean value based upon the number of experiments indicated in parentheses. If 4 or more experiments were performed a standard deviation was determined. In some experiments fluorescence quenching was observed but the resolution of the data was not sufficient to accurately determine a K_d value (K_d > 10 μM). [b]The experiments were performed as described in Methods using the toxin rate inhibition assay. The K_{d_I} and $K_{d_{II}}$ values expressed and error indicated result from least squares regression analysis of the data. In some experiments the probe concentrations used were not great enough to accurately determine the K_{d_I}.

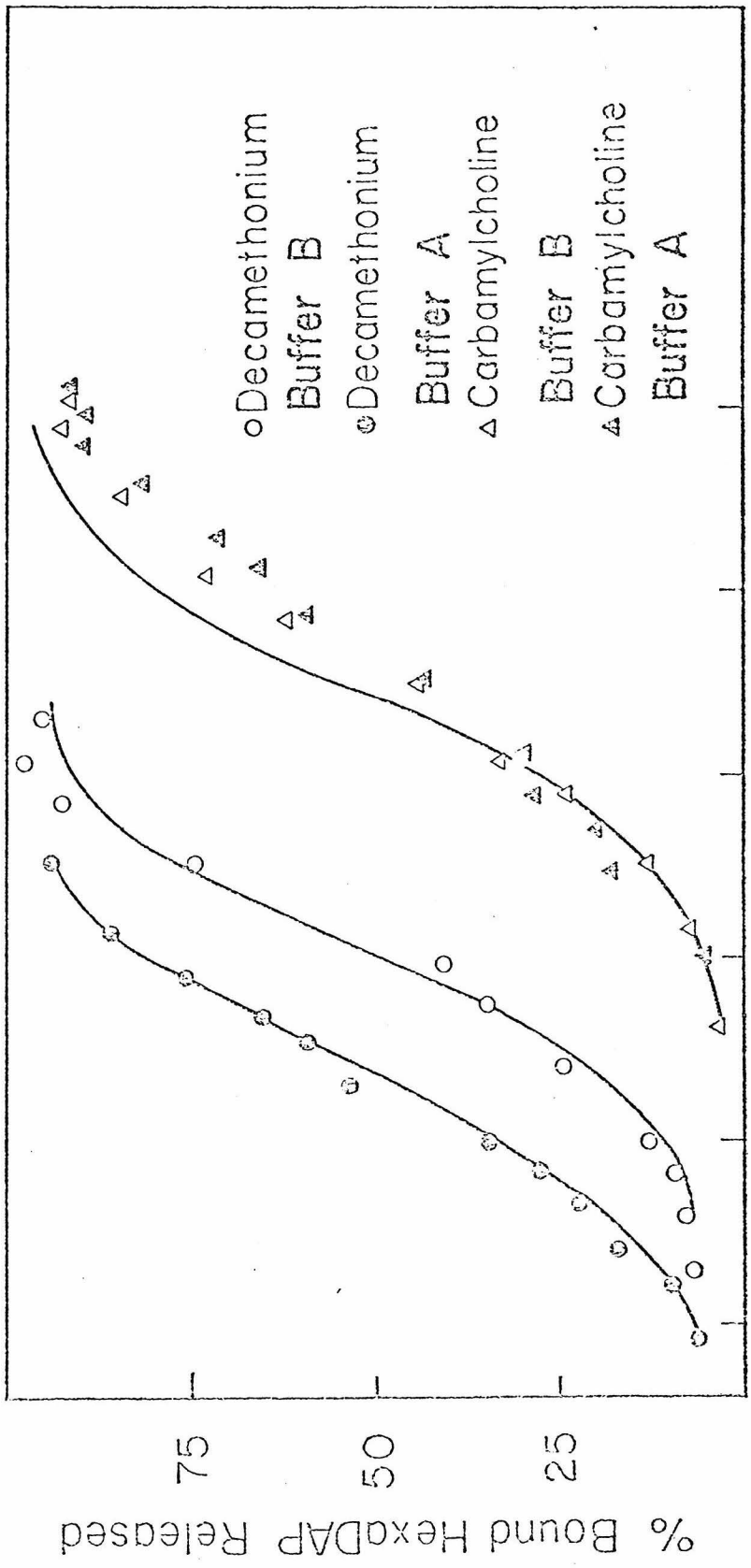
with lower affinity.

Figure 7 illustrates that the affinity of the receptor for OAP decreased dramatically as the ionic strength of the buffer increased. In Buffer A, Kd = 0.26 μM, and in Buffer B, Kd = 0.95 μM. A summary of the direct binding data of the bifunctional probes obtained at equilibrium using both Buffers A and B is presented in Table III. These data indicate that all bifunctional probes tested appeared to bind with 4-7 fold lower affinity in the buffer containing high concentrations of NaCl (Buffer B). Martinez-Carrion and Raftery (64) proposed that this effect was not solely related to ionic strength, for they observed that divalent cations appeared to inhibit DAP binding with 30 fold greater affinity than did monovalent cations. Similarly, Schmidt and Raftery (72) observed that divalent cations slowed the rate of ^{125}I-α-BuTx binding to the purified receptor with 30 fold greater affinity than did monovalent cations, whereas anions had no effect. They proposed that cations either indirectly affect the tertiary structure of the receptor at the DAP and α-BuTx binding site, or that cations directly bind to and occupy anionic sites critical to the binding of DAP and α-BuTx.

Fluorescent Probe Displacement Studies. The effect of cholinergic ligands on bifunctional probe binding was investigated. The percent of bound HexaDAP released versus log free inhibitor concentration is shown in figure 9. Decamethonium appeared to

Figure 9.

Plot of the percentage of bound HexaDAP released versus the log free inhibitor concentration, [I]. When Buffer A was used the total concentration of HexaDAP was 0.125 μM and the purified receptor concentration was 0.17 μM in α-BuTx binding sites. When Buffer B was used the total HexaDAP concentration was 0.36 μM and the receptor concentration was 0.52 μM in α-BuTx binding sites. The solid lines drawn represent the best fit assuming competitive inhibition when Ki = 0.4 μM (●), Ki = 2.2 μM (○), and Ki = 40 μM (△,▲).


% Bound HexaDAP Released
75
50
25
-1
0
1
2
3
4
Log [I] μM
o Decamethonium
Buffer B
o Decamethonium
Buffer A
Δ Carbamylcholine
Buffer B
Δ Carbamylcholine
Buffer A

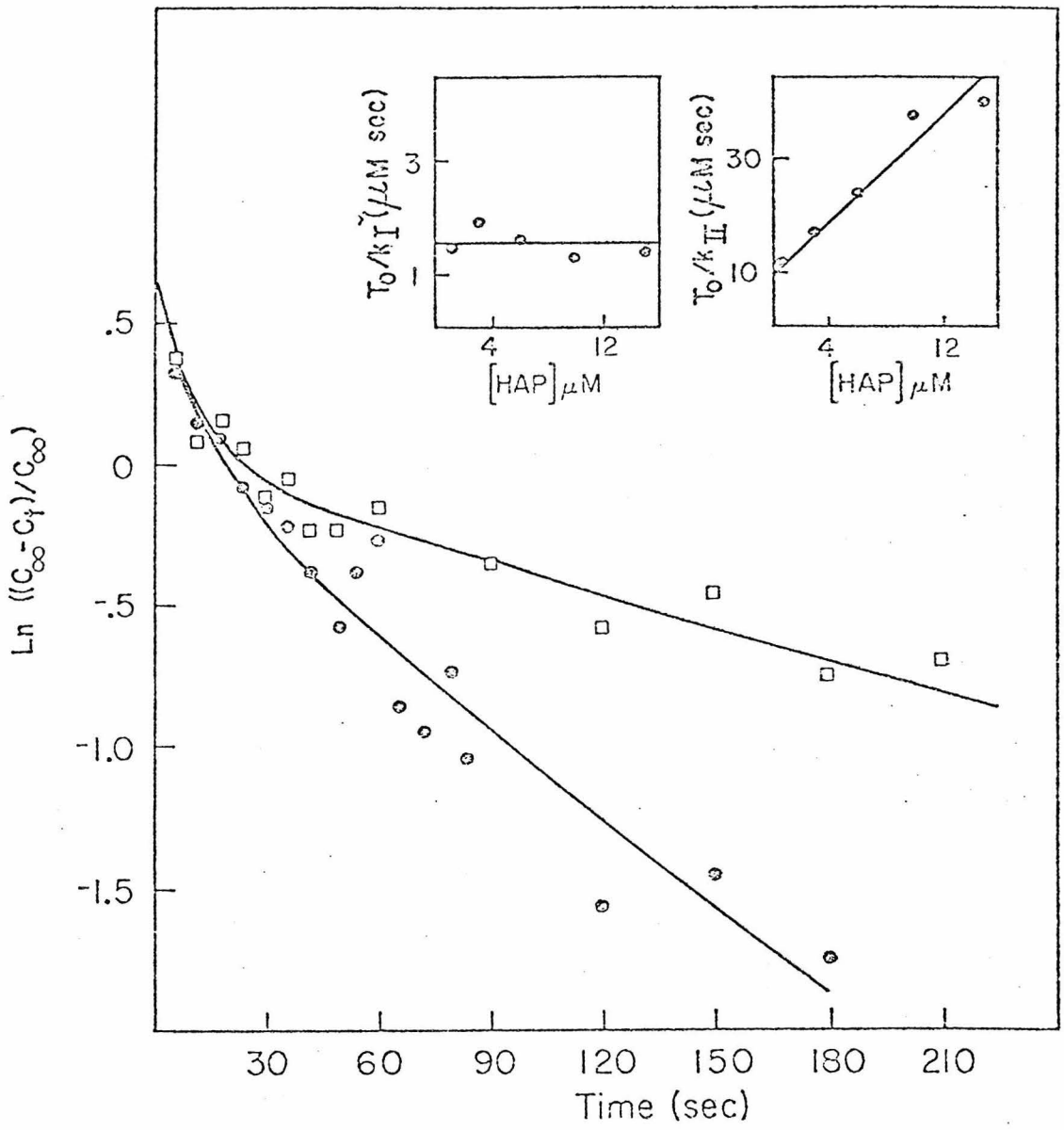
inhibit HexaDAP binding with greater affinity than did carbamylcholine. Further, figure 9 shows that decamethonium inhibited HexaDAP binding with greater affinity in Buffer A (Ki = 0.4 μM) than Buffer B (Ki = 2.2 μM), whereas carbamylcholine inhibited HexaDAP binding with the same apparent affinity in Buffers A and B. These data indicate that high concentrations of Na^+ may inhibit decamethonium binding, whereas carbamylcholine binding is unaffected. In addition, the decamethonium inhibition curves were sigmoidal in shape and could be fit assuming competitive inhibition. The carbamylcholine curves were shallower in slope and could not be fit assuming competitive inhibition. Equation 2 describes adequately the carbamylcholine inhibition data assuming that Ki'/Ki = 50. Therefore, we calculate that appreciable amounts of receptor-HexaDAP-carbamycholine ternary complexes form, whereas only binary Deca-receptor or probe-receptor complexes form. Similar data were obtained using carbamylcholine and decamethonium as inhibitors when other bifunctional probes were substituted for HexaDAP.

Toxin Binding Kinetics. Previously, Raftery et al. (61,62) and Reed et al. (63) demonstrated that high concentrations of DAP reduced greatly the rate of ^{125}I-α-BuTx binding to the "slow" site whereas toxin binding to the "fast" site was essentially unaffected. They postulated that DAP binds with at least a hundred fold greater affinity to the "slow" than to the "fast" site. Figure 10 shows

Figure 10.

Plot illustrating the rate of ^{125}I-α-BuTx binding to the purified receptor in the absence (●) and presence (□) of 15 μM HAP. Initially, the total receptor concentration was 15 nM in α-BuTx binding sites and the toxin concentration was 120 nM. The solid lines were calculated from a least squares regression analysis of the data. Buffer B was used.

Inserts: Left: A replot of To/k_I versus HAP concentration. Right: A replot of To/k_{II} as a function of HAP concentration. The calculated $Kd_{II} = 4.2 \pm 0.5$ μM.


Ln ((C∞-Cf)/C∞)
.5
0
-.5
-1.0
-1.5
30
60
90
120
150
180
210
Time (sec)
T0/kI (μM sec)
3
1
4
12
[HAP] μM
T0/kII (μM sec)
30
10
4
12
[HAP] μM

a similar experiment using HAP. Only the rate of toxin binding to the "slow" site was greatly reduced by HAP. Table III summarizes the Kd_I and Kd_{II} values calculated for HAP and the other synthetic probes. Since the Kd_{II} values obtained using this kinetic assay closely approximate the Kd values obtained when we performed the fluorimetric assay using Buffer B, we conclude that HAP, OAP, DAP, and DoDAP bind with 10-100 fold greater affinity to the "slow" rather than to the "fast" site, whereas BAP and the monofunctional ligands bind with only 2-3 fold greater affinity to the "slow" site. Other evidence indicates that TetraDAP, HexaDAP, and OctaDAP bind with at least 10 fold greater affinity to the slow than to the fast site.

DISCUSSION

The purified receptor used here has two equal populations of ligand and toxin binding sites. Cholinergic ligands bind with slightly greater affinity to the "slow" than to the "fast" toxin binding site (61,62,63). Since all of the synthetic bifunctional probes bind with substantially greater affinity to the "slow" than to the "fast" site (with the possible exception of BAP), they serve as useful tools to examine the ligand binding topography at this specific class of sites.

If the synthetic bifunctional probes interacted strongly with only one subsite, the mono- and bifunctional probes should

bind with similar affinities. However, we observe that the receptor binds the bifunctional probes with substantially greater affinity than the monofunctional probes. We calculate that the free energy of binding for 3-aminopyridinium-1-hexane is -5.4 kcal per mole assuming $\Delta G = -RT\ln(1/Kd)$. In contrast, the free energy of binding for DoDAP using Buffer B is -9.1 kcal/mole. The simplest interpretation of these data is that the receptor interacts with both ends of a bifunctional probe such as DoDAP. We propose that monofunctional probes and bifunctional probes interact with a single site, subsite A, whereas DoDAP interacts with two sites, subsite A and subsite B. Further, figure 8 indicates that the receptor binds the synthetic bifunctional probes with increasing affinity as the number of methylene groups (n) increases from 4 to 12 and decreases from n = 18 to n = 16. The simplest interpretation of these data is that subsites A and B are separated by a fixed distance, such that only the longer chain bifunctional probes (n = 12, 14, and 16) interact strongly with both subsites. Since we calculate that the distance between pyridinium nitrogens in TetraDAP when it is extended fully is 19 Å, subsites A and B could be as distant as 19 Å.

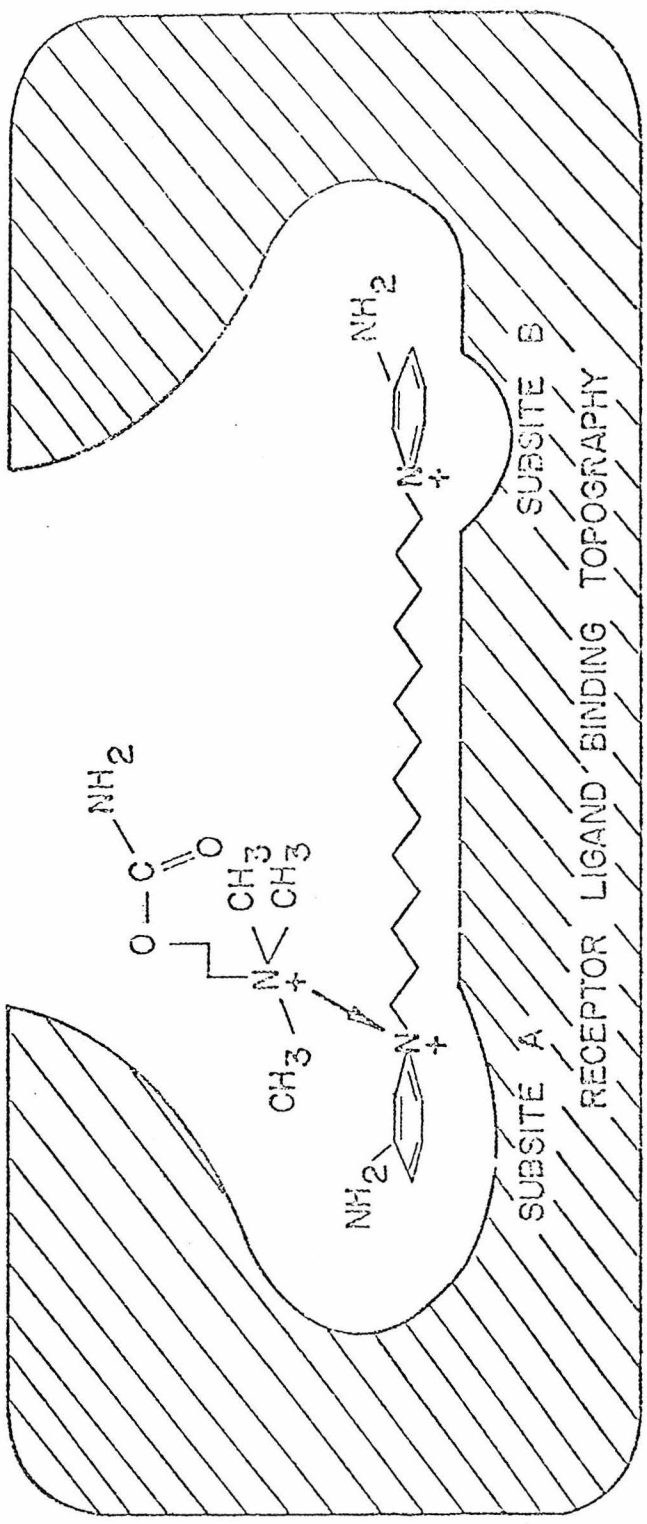
While the energetics of binding vary for the bifunctional probes as a function of methylene chain length, each bifunctional probe is inhibited by high concentrations of decamethonium as well as carbamylcholine. Figure 9 shows that decamethonium

inhibits HexaDAP binding competitively, whereas the carbamylcholine data were fit best assuming that significant amounts of receptor-HexaDAP-Carb ternary complex form. These data indicate that Deca interacts strongly with a site(s) important in the binding of bifunctional probe whereas carbamycholine interacts weakly with this site(s). Since we propose that all of the bifunctional probes interact strongly with subsite A, decamethonium must also interact strongly with subsite A, whereas carbamylcholine may interact weakly with this site. Also, Table III shows that the receptor binds the bifunctional probes with 4-7 fold greater affinity in Buffer A than Buffer B. Similarly, figure 9 shows that decamethonium inhibits HexaDAP binding competitively with greater affinity in Buffer A (Ki = 0.4 M) relative to Buffer B (Ki = 2.2 M). These data indicate that high concentrations of Na^+ may greatly reduce bifunctional probe-receptor as well as decamethonium-receptor interactions at subsite A.

A ligand binding scheme consistent with these data is shown in figure 11. All synthetic mono- and bifunctional probes as well as decamethonium interact strongly with subsite A, possibly through van der Waal's and Coulombic interactions. In addition, long bifunctional probes such as TetraDAP interact strongly with subsite B. High concentrations of Na^+ reduce receptor-bifunctional probe as well as receptor-decamethonium interactions at subsite A because Na^+ may bind to and neutralize an anionic site at subsite

Figure 11.

Schematic diagram illustrating the ligand binding topography of the "slow" site.


RECEPTOR LIGAND BINDING TOPOGRAPHY
SUBSITE A
SUBSITE B
NH2
NH2
CH3
CH3
CH3
O
O
C
O
NH2

A, or Na^+ may affect the receptor tertiary structure at subsite A. Similarly, high concentrations of carbamylcholine may reduce receptor-bifunctional probe interactions at subsite A because of steric effects (subsite A and the carbamylcholine binding site may overlap partially), or because carbamylcholine affects the receptor tertiary structure at subsite A.

A similar type of binding scheme has been postulated based on in vivo experiments using decamethonium analogues. Barlow and Zoller (73) observed that the ability of bisonium ligands to induce contraction in a chick biventer cervicus preparation did not change as the number of methylene groups (n) between the two trimethylammonium moieties was varied from 10 to 16. However, the relative blocking potency assayed using a rat diaphragm preparation was maximal when n = 15 and sharply declined as n was increased from 18 to 19 or decreased from 11 to 10. Similarly, Paton and Zaimis (74) observed a maximum in blocking potency using a cat tibialis preparation when n = 10 and n = 16, and the blocking potency rapidly decreased when n was increased from 18 to 19 or decreased from 9 to 8. These results indicate that while the depolarizing activity of bisonium ligands does not vary sharply as a function of the number of methylene groups (n), the neuromuscular blocking activity does. As a result of these data, Khromov-Borisov and Michelson (75) proposed that when decamethonium

binds to the receptor, each positively charged trimethylammonium group interacts with fixed anionic sites. Since they postulated that decamethonium bound to the receptor in a fully extended conformation, they calculated that the distance between nearest neighbor anionic sites was 14 Å. In addition, two other anionic sites of physiological importance were separated by 20 Å.

Figure 11 illustrates that the "slow" site binds TetraDAP with high affinity when the bifunctional probe is in its fully extended conformation. Since we calculate that the distance between pyridinium nitrogens in TetraDAP is 19Å when it is extended fully, the distance between subsites A and B in figure 11 is approximately 19 Å. However, we are not certain that our fluorescent decamethonium analogues bind to the "slow" site in a fully extended conformation, consequently the distance between subsites A and B may be less than 19 Å. Nonetheless it is interesting that the purified receptor binds our fluorescent decamethonium analogues with increasing affinity as the number of methylene groups increased from 4 to 12 and decreased from 18 to 16. If the blocking potency of these analogues on the Torpedo electroplaque declined sharply as n increased from 16 to 18 or decreased from 12 to 4, it would indicate that the "slow" site may contain the antagonist binding site.

CHAPTER III

EFFECTS OF VARIOUS COMPONENTS PURIFIED FROM THE VENOM OF *BUNGARUS CAERULEUS* ON THE LIGAND BINDING PROPERTIES OF THE ACETYLCHOLINE RECEPTOR

Numerous neurotoxins have been isolated from various snake venoms and frog skins. Their characteristic mode of action *in vivo* is to block the neuromuscular junction. α-BuTx, purified from the venom of *Bungarus multicinctus*, is a basic polypeptide of molecular weight 8,000 daltons (15,16). This toxin binds to the AcCh receptor in an essentially irreversible manner thereby preventing the binding of AcCh to this postsynaptic site (17). β-BuTx, also purified from *Bungarus multicinctus* venom, is a basic polypeptide of molecular weight 21,000 daltons (13). This toxin stimulates initially, then inhibits transmitter release by an unknown mechanism (76). Histionicotoxin, which is an alkaloid purified from the frog *Dendrobates histrionicus*, is postulated to be a specific probe for the ion translocation device regulated when AcCh binds to the receptor (19). Kato and Changeux obtained data consistent with this hypothesis when they observed that micromolar concentrations of histrionicotoxin block the carbamylcholine induced depolarization of the *Electrophorus* electroplaque, whereas ^{3}H-AcCh binding to membrane preparations enriched for receptor is unaffected (20). Recently, Bon and Changeux (77) purified a polypeptide neurotoxin from the venom of *Bungarus caeruleus*. Similar to histrionicotoxin, this polypeptide blocked the carbamylcholine induced depolarization of the *Electrophorus* electroplaque but did not affect ^{3}H-AcCh binding to receptor enriched membrane preparations. Since such a polypeptide might serve as a valuable

probe for the ion translocation device, we attempted to purify and characterize this toxin from Bungarus caeruleus venom.

MATERIALS

Lyophilized Bungarus caeruleus venom was obtained from Sigma Chemical Co. (Lot # 125C-0088) and Miami Serpentarium (Lot # BC 655BZ-1). Lyophilized Bungarus multicinctus venom was obtained from Sigma Chemical Co. and ^{125}I-α-BuTx was prepared using the method of Clark et al. (16). Carbamylcholine chloride and acetylthiocholine iodide were obtained from Aldrich Chemical Co. Soybean phosphatidylcholine and phospholipase A_2 were obtained from Sigma Chemical Co. All other chemicals used were reagent grade and obtained from commercial sources. CM-52 cation exchange resin was obtained from Whatman and QAE-Sephadex A-50 anion exchange resin was obtained from Pharmacia Fine Chemicals. Male adult Swiss-Webster mice were obtained from Simonsen Laboratories Inc.

METHODS

Crude venom (500 mg) was extracted for 30 minutes with 50 mM Tris-HCl (pH 8.5). Insoluble material was pelleted by centrifugation for 10 minutes at 10,000 rpm, the supernatant removed and pellet reextracted. The supernatants were combined and applied to a QAE-Sephadex A-50 column (20 x 2 cm) as described by Bon and Changeux (77). After the basic fractions passed through

the column the acidic fractions were eluted with a linear salt gradient of 0-0.25 M NaCl in 50 mM Tris-HCl (pH 8.5). Fractions were pooled, dialyzed extensively against water, and lyophilized.

The lyophilized basic fraction was resuspended in 50 mM Na phosphate (pH 6.8) and applied to a CM-52 column (25 x 3.5 cm). The fractions were eluted with a linear salt gradient of 0-0.5 M NaCl in 50 mM Na phosphate (pH 6.8). Fractions were pooled, dialyzed extensively against H_2O, and lyophilized.

Column eluates were assayed for NaCl concentration using a Radiometer CDM-3 conductivity meter, u.v. absorbance at 260 and 280 nm, and protein concentration by the method of Lowry et al. (56) using BSA as standard. In the case of the CM-52 column, but not the QAE-Sephadex A-50 column, the absorbance values and protein concentration gave identical profiles.

The pooled fractions were assayed for toxicity, esterase activity, phospholipase activity, and homogeneity. Toxicity was assayed in white mice (78). Concentrated protein solutions were diluted into pyrogen free NaCl. After the intraperitoneal (i.p.) injection of 200 µl of sample, the time of death, weight of the dead mouse, and death symptoms were recorded. If a mouse was to die it did so within 24 hours after injection.

Esterase activity was determined by the method of Ellman et al. (79). 0.5 mM Acetylthiocholine was used as substrate. The buffer contained 0.33 mM DTNB and 0.1 M Na phosphate (pH 8.0).

Microgram quantities of protein were added, mixed, and the amount of thiocholine released determined as a function of time by measuring the absorbance at 412 nm.

Phospholipase activity was determined using the method of Strong et al. (14). 2 mM Soybean phosphatidylcholine was used as substrate. The buffer contained 3 mM Na deoxycholate, 100 mM NaCl, and 10 mM $CaCl_2$. One tenth ug quantities of protein were injected and the amount of fatty acid released titrated as a function of time at pH 8.0 and 37°C. using a pH-stat (Radiometer TTT-3 Autotitrator).

Homogeneity was assessed by SDS gel electrophoresis in 8 M urea (80). Ten ug quantities of protein were loaded onto each gel; the gels were composed of 10% polyacrylamide and the ratio of acrylamide to bis was 32:1 (w:w).

Membrane preparations containing receptor were prepared using the membrane fractionation scheme shown in figure 2. These membrane fragments were either used directly or purified further on sucrose density gradients. Since Reed et al. (68) observed that membranes enriched with AcCh receptor have a density of 1.18 mg/ml, large quantities of these fragments are prepared on a continuous 30-50% (w:v) sucrose gradient in a Beckman Ti-15 Zonal rotor. The rate of ^{125}I-α-BuTx binding to receptor enriched membrane preparations was determined using the method of Lee et al. (25). Membranes were added to Torpedo Ringers and incubated for 10 minutes with the pooled fractions. Then excess ^{125}I-α-BuTx

was added under the conditions described in figure 15 or 16. At given time intervals, 100 µl aliquots were withdrawn and the amount of ^{125}I-α-BuTx-receptor complex determined (59).

The AcCh receptor was solubilized and purified using the method of Schmidt and Raftery (40). The rate of ^{125}I-α-BuTx binding to the purified receptor was determined using the method of Raftery et al. (61,62) and Reed et al. (63).

RESULTS

Chromatographic Separation and Purification of Toxins.

The elution profile of the crude venom fractionated on a QAE-Sephadex A-50 anion exchange column is shown in figure 12. The five peaks were pooled as indicated. Peak 1 had substantial toxicity; 1 ug injected i.p. was lethal in mice. Peaks 2,3,4, and 5 were not highly toxic; 100 ug of injected protein from any of these peaks was nonlethal in mice. While peak 2 contained little protein, it did contain a component which permeated dialysis tubing. This low molecular weight material had a u.v. absorbance maximum at 254 nm and was not highly toxic. Peaks 3 and 4 released 2 micro-equivalents of fatty acid per minute per ug of protein. Peak 5 hydrolyzed $\simeq 10^{-3}$ moles of acetylthiocholine per minute ug of protein.

Figure 12.

Anion exchange chromatography of crude Bungarus caeruleus venom on QAE-Sephadex A-50 in 50 mM Tris-HCl (pH 8.5). Fraction size was 3 ml.

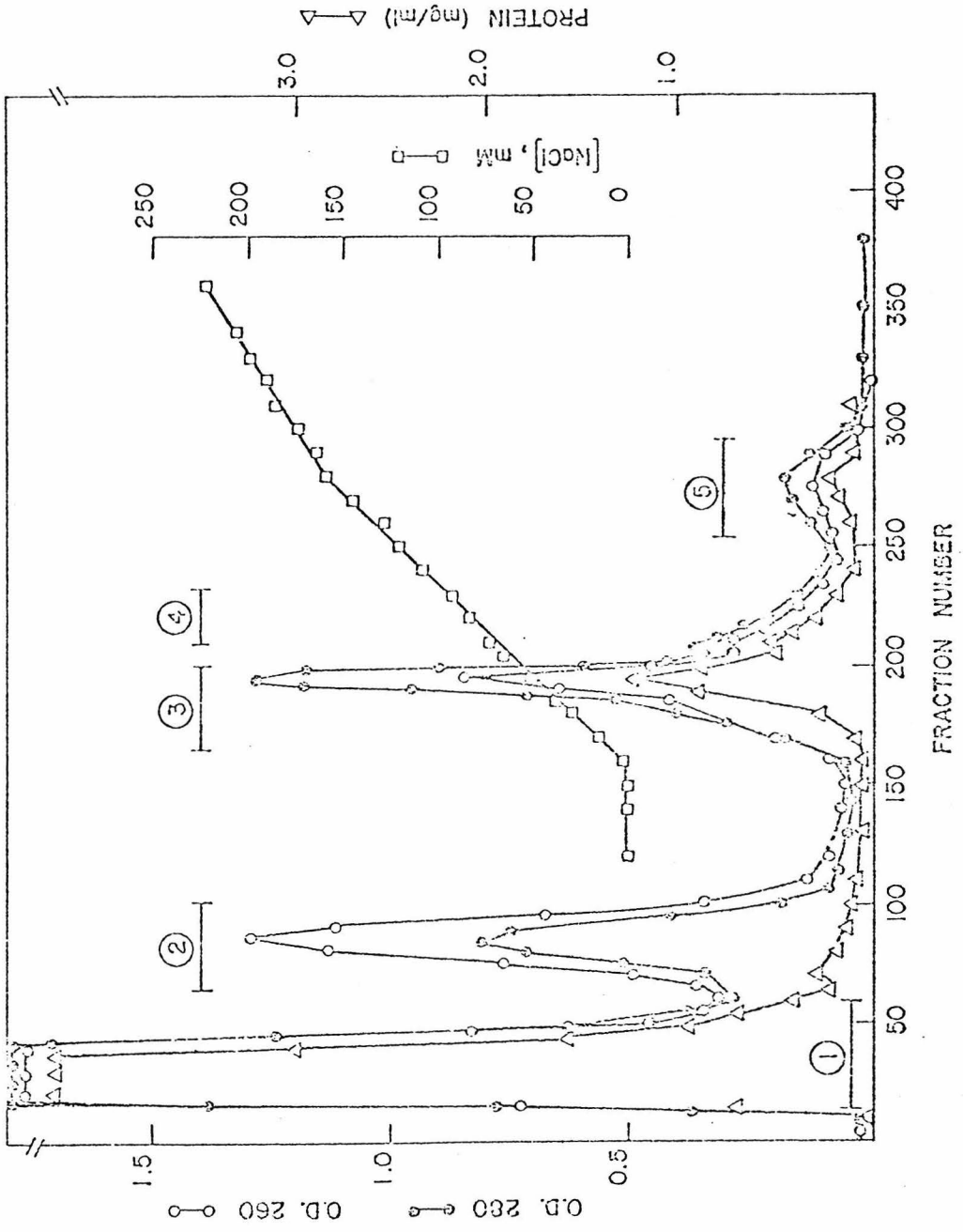

PROTEIN (mg/ml)
3.0
2.0
1.0
[NaCl], mM
250
200
150
100
50
0
O.D. 280
O.D. 260
1.5
1.0
0.5
1
2
3
4
5
50
100
150
200
250
300
350
400
FRACTION NUMBER

Figure 13 shows the elution profile of peak 1 chromatographed on a CM-52 column. The 10 resultant peaks were pooled as indicated. Peaks B,C,I, and J were found to be toxic. Material from peaks I and J injected i.p. was lethal at a 0.1 ug dose. Material from peaks B and C was lethal at a 10 ug dose, and 50 ug of material from peaks A,D,E,F,G, and H was not lethal. Peak E released 0.8 microequivalents of fatty acid per minute ug of protein. The homogeneity of the pooled protein fractions was determined by polyacrylamide gel electrophoresis in the presence of SDS and 8 M urea. Since in the absence of reducing agent a single major polypeptide band was observed for each of the basic fractions, these pooled fractions may be 90% pure.

Lethality of Toxic Fractions in Mice. The LD_{50} in mice of peaks B,C,I, and J was determined as described by Chang and Lee (78). The data were analyzed using the method of Litchfield and Wilcoxin (81). Table IV shows that peaks I and J were highly toxic, whereas a 50-100 fold greater dose of protein from peaks B or C was required for lethality. Mice which died following injection of I or J were hyperirritable initially; flaccid paralysis of the extremities developed progressively, followed by rapid and shallow breathing; death appeared to be due to respiratory failure. Mice which died following injection of B or C showed no hyperirritability and developed flaccid paralysis just prior to death. The death symptoms and LD_{50} values obtained using peaks B or C are similar to those

Figure 13.

Cation exchange chromatography of basic components from peak 1 of figure 12 on CM-52 in 50 mM Na phosphate (pH 6.8). Fraction size was 7.5 ml.

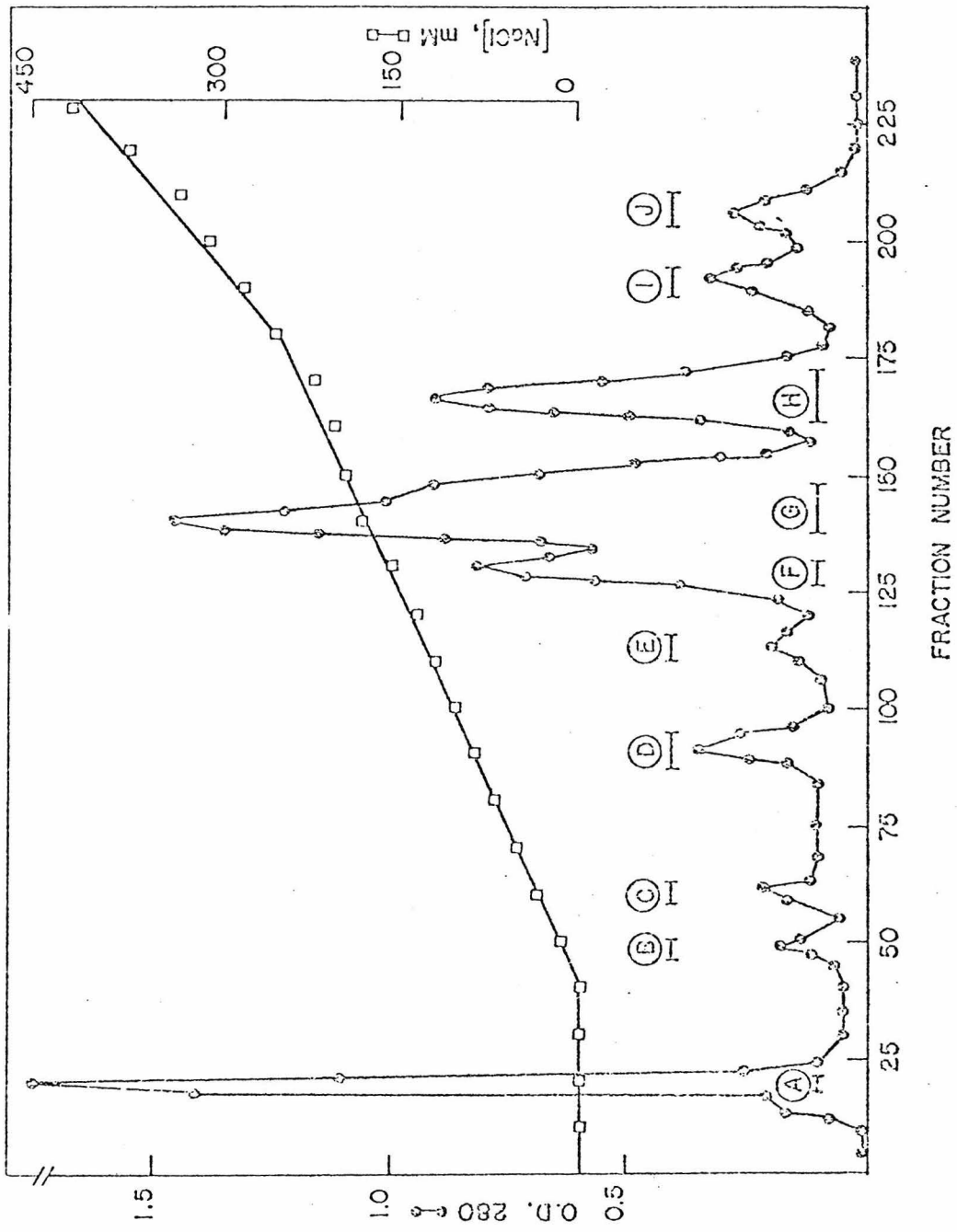

[NaCl], mM
450
300
150
0
O.D. 280
1.5
1.0
0.5
25
50
75
100
125
150
175
200
225
FRACTION NUMBER
A
B
C
D
E
F
G
H
I
J

TABLE IV

LD_{50} AND SLOPE FUNCTIONS OF TOXIC FRACTIONS
BUNGARUS CAERULEUS VENOM

FRACTION	LD_{50} (i.p.) IN MICE (NG/ G) [a]	SLOPE FUNCTION[b]
Crude Venom	42.2 (28 - 64)	1.92
B	176 (106 - 292)	1.92
C	258 (167 - 400)	1.63
I	3.3 (2.1 - 7.3)	1.84
J	2.6 (1.7 - 4)	1.50

[a] Figures in parentheses denote 95% confidence limit.

[b] Slope function = $\frac{1}{2}$ $(LD_{84}/LD_{50} + LD_{50}/LD_{16})$.

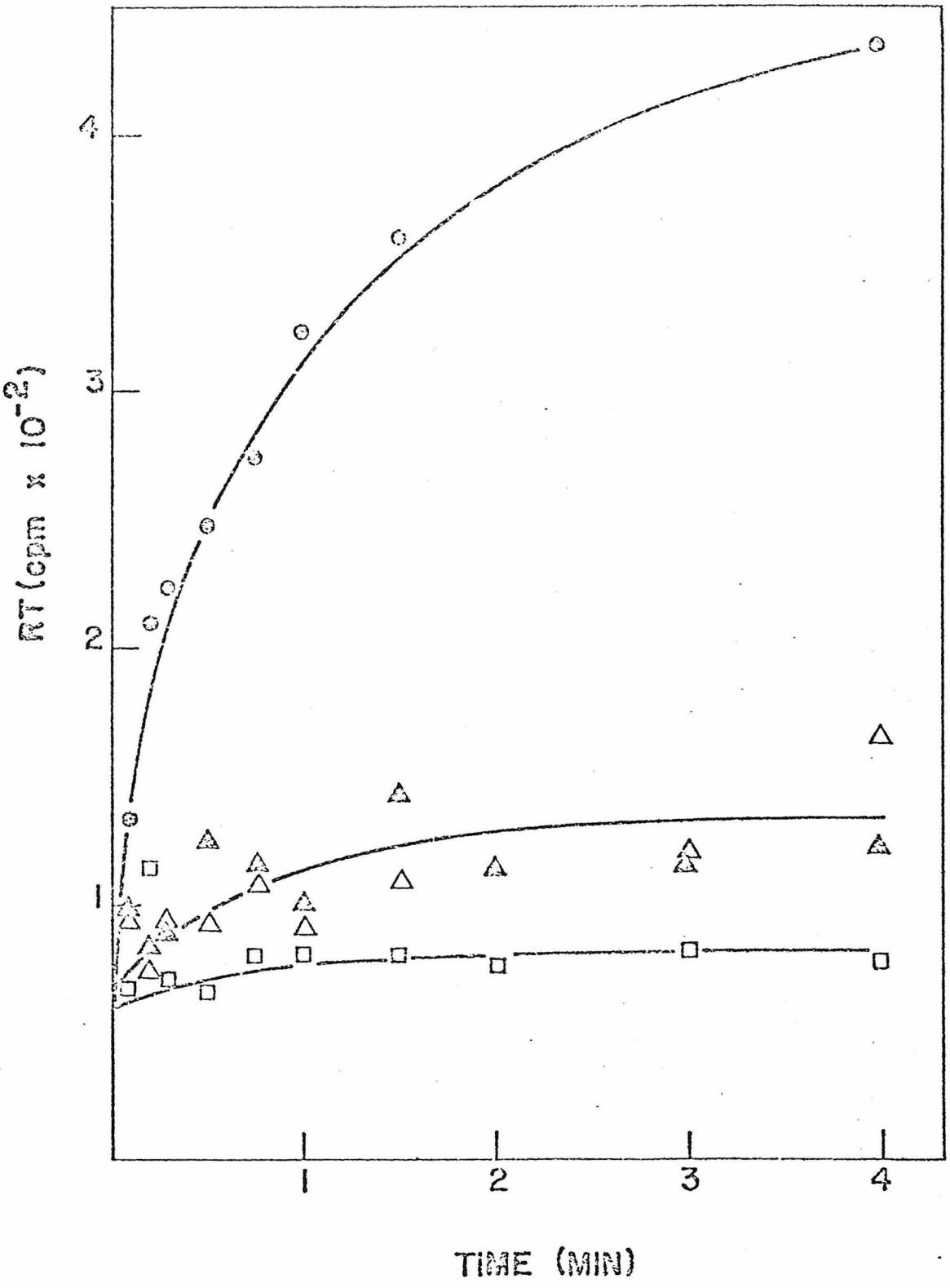
observed for α-BuTx (78). The death symptoms resulting from injection of protein from peaks I or J are similar to those observed for β-BuTx (78), but the LD_{50} values for peaks I and J are an order of magnitude lower than that for β-BuTx.

While this work was in progress, Lee et al. (82) fractionated crude Bungarus caeruleus venom obtained from Miami Serpentarium on a CM-52 column using a linear 0.05 (pH 5.8) to 0.5 M (pH 6.8) ammonium acetate gradiant. They obtained two toxic fractions at 0.1 M NH_4Ac which gave an $LD_{50} \simeq 0.2$ ug/g of mouse. Two other toxic components were resolved at 0.4 M NH_4Ac and gave an $LD_{50} \simeq 0.01$ ug/g. These results are very consistent with those presented here. Further, Lee et al. (82) observed that the two toxins with the $LD_{50} \simeq 0.2$ ug/g were similar in behavior to the postsynaptic toxin α-BuTx when applied to a chick biventer cervicus preparation. The other two toxins, when applied to a chick biventer cervicus preparation, appeared to act as presynaptic toxins similar to β-BuTx.

Biochemical Studies. Further proof that peaks B and C contain postsynaptic neurotoxins, similar to α-BuTx is provided by figure 14. If the purified receptor (5.0 nM in ^{125}I-α-BuTx binding sites) was pretreated with 0.2 ug/ml of α-BuTx for 10 minutes (this represents a five fold excess of α-BuTx relative to the number of sites) the rate of ^{125}I-α-BuTx binding to the receptor is reduced at least 20 fold. Similarly, if the receptor

Figure 14.

Plot illustrating the rate of ^{125}I-α-BuTx binding to the purified AcCh receptor. ^{125}I-α-BuTx (62 nM) was added to receptor (5 nM in α-BuTx binding sites) after the receptor was incubated for 10 minutes with no additions (●), 0.2 ug/ml peak B (△), 0.2 ug/ml peak C (▲), and 0.2 ug/ml α-BuTx (□). The buffer contained 0.1% Triton X-100, 10 mM Na phosphate, and 100 mM NaCl (pH 7.25).


RT (cpm x 10^{-2})
4
3
2
1
1
2
3
4
TIME (MIN)

was pretreated with 0.2 ug/ml of peak B or C for 10 minutes the rate of ^{125}I-α-BuTx binding to the receptor is reduced approximately 10 fold. Since α-BuTx is postulated to bind to the cholinergic ligand binding site in an essentially irreversible manner (17), these results indicate that peaks B and C bind to this site with high affinity.

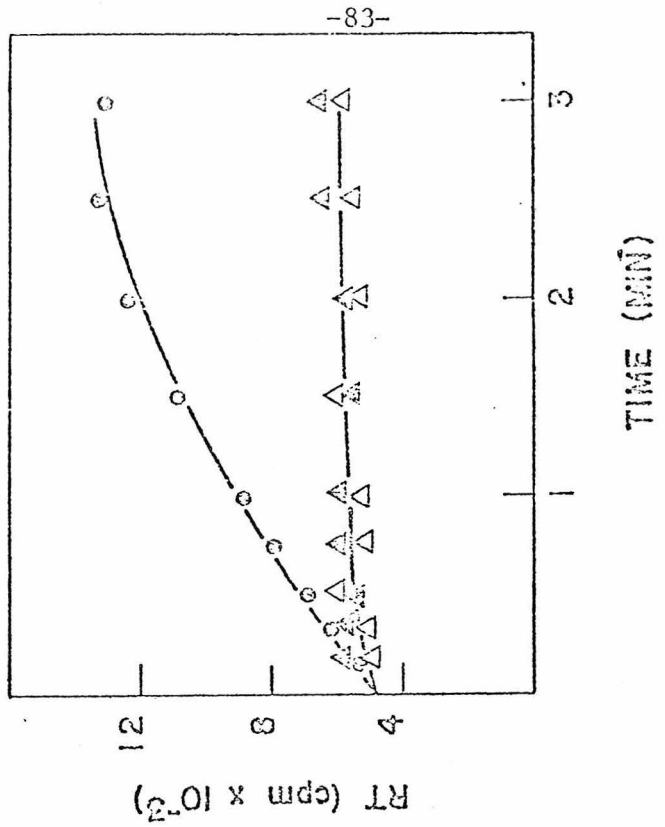
Also, we observed that treatment of receptor enriched membrane preparations with peak 4 increased the affinity of the receptor for carbamylcholine. Figure 15 (left) shows that 1 μM Carb causes a time dependent change in membranes enriched with receptor such that the rate of ^{125}I-α-BuTx binding is reduced greatly. Weber et al. (26) and Lee et al. (25) proposed that in the presence of carbamylcholine, the receptor undergoes a time dependent change in conformation such that it binds carbamylcholine with greater affinity. Using 1 μM Carb, the half time of this process is 3 minutes (25). Figure 15 (right) shows that treatment of receptor enriched membrane preparations with peak 4 does not affect the rate of ^{125}I-α-BuTx binding to the receptor, however, the rate of ^{125}I-α-BuTx binding in the presence of 1 μM Carb added simultaneously is reduced greatly. It is possible that peak 4 affects the receptor conformation such that the receptor binds carbamylcholine with greater affinity.

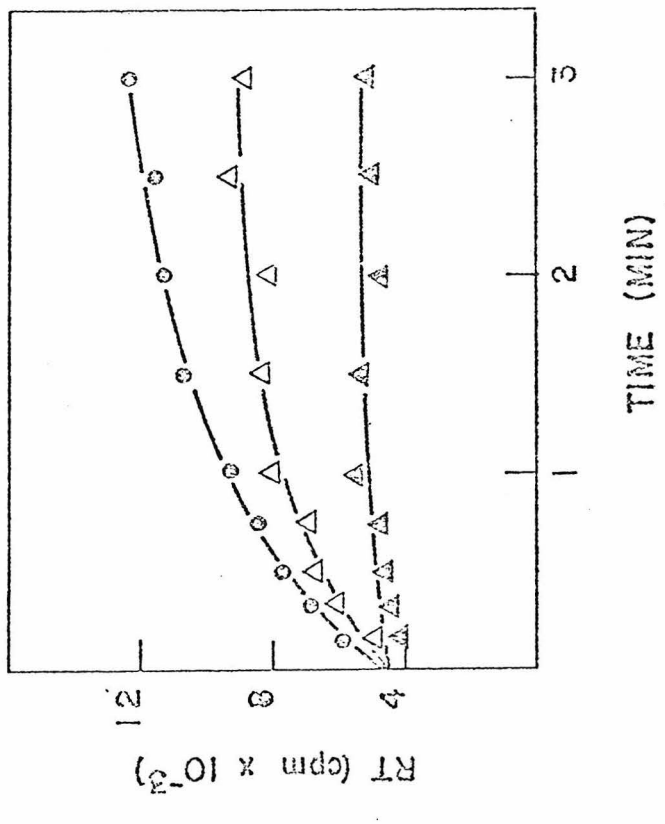
Since peak 4 has substantial A-type phospholipase activity (W. Wu and M.A. Raftery, unpublished) the receptor enriched

Figure 16.

Left: Figure illustrates the rate of ^{125}I-α-BuTx binding to membrane fragments enriched with receptor. ^{125}I-α-BuTx (0.33 μM) was added to receptor (0.071 μM in α-BuTx binding sites) with no additions (●), with 1 μM Carb (△), and after the receptor was incubated with 1μM carbamylcholine for 10 minutes (▲). The buffer contained 10 mM HEPES-NaOH, 5 mM KCl, 2 mM $MgCl_2$, 4 mM $CaCl_2$, and 250 mM NaCl (pH 7.5).

Right: Same as above except that the receptor enriched membrane preparations were preincubated with 5 ug/ml of peak 4 for 10 minutes.


RT (cpm x 10⁻³)
12
8
4
1
2
3
TIME (MIN)


RT (cpm x 10⁻³)
12
8
4
1
2
3
TIME (MIN)

membrane preparations may bind carbamylcholine added simultaneously with greater affinity due to phospholipase A enzymatic activity. In this regard, if membrane fragments enriched with receptor were treated with 1 ug/ml Crotalus terrificus phospholipase A, the receptor bound Carb added simultaneously with increased affinity. Further, figure 16 (left) shows that as little as 12 ng/ml of peak 4 in the presence of Ca^{++} affects the receptor so that it binds Carb added simultaneously with high affinity, however, figure 16 (right) shows that peak 4 in the absence of Ca^{++} has no effect and as a result the receptor binds Carb added simultaneously with low affinity. Since we observed that the phospholipase activity of peak 4 was reduced a hundred fold if Ca^{++} was omitted from the buffer and 1 mM EGTA included, we propose that exogenous A-type phospholipase activity may affect the receptor conformation so that membrane preparations enriched with receptor bind carbamylcholine added simultaneously with greater affinity.

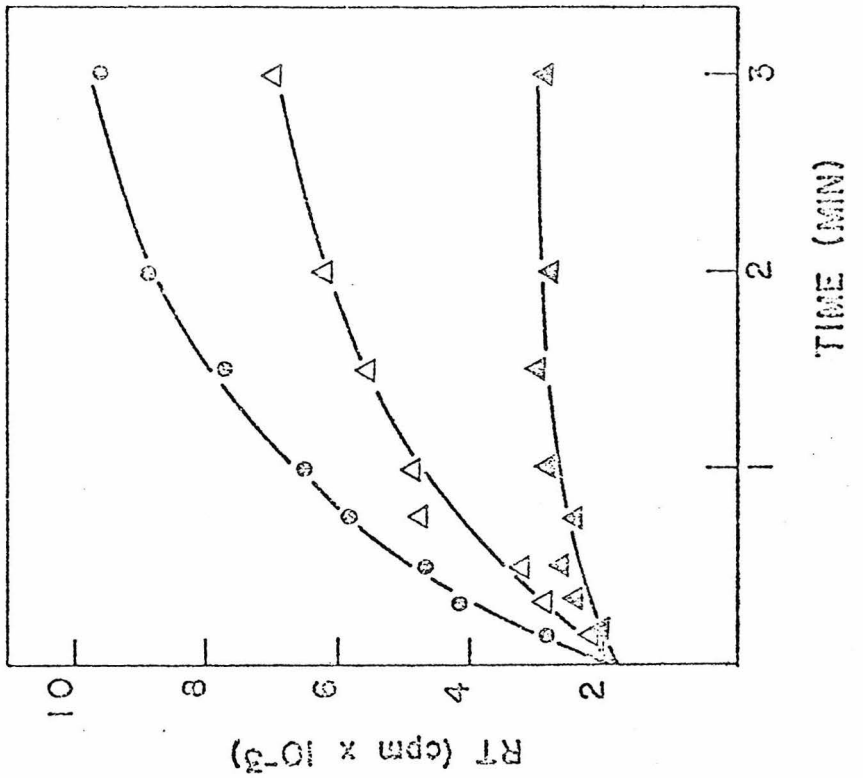
DISCUSSION

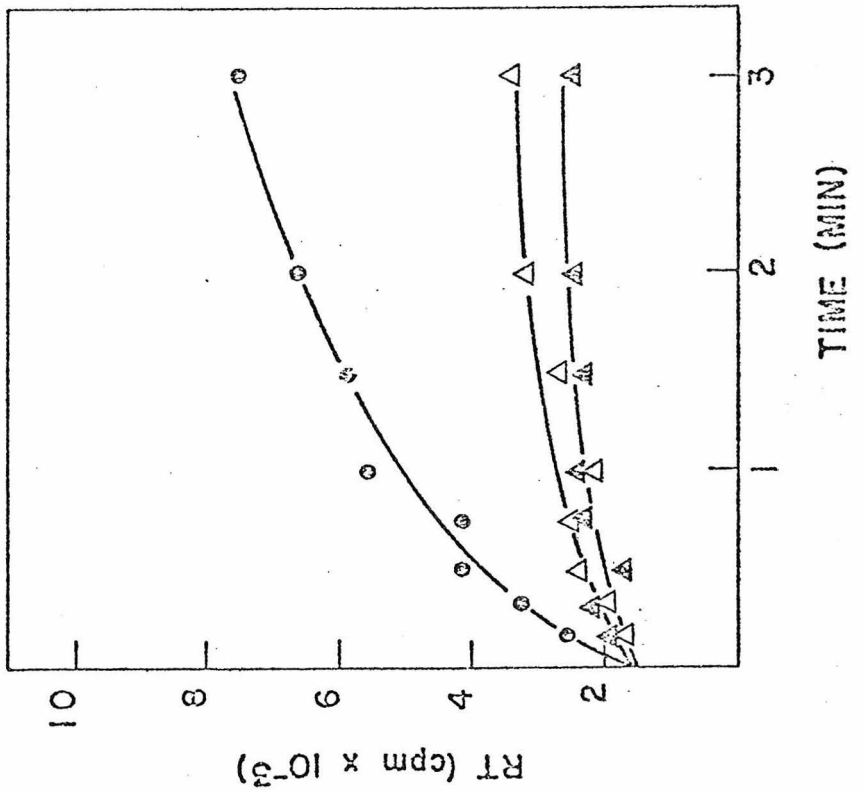
Using the procedure described here, we found four protein components in Bungarus caeruleus venom which are lethal in mice. These results are consistent with those of Lee et al. (82). They isolated two components from Bungarus caeruleus venom which act as postsynaptic toxins when assayed using a chick biventer cervicus preparation. We observed that if purified receptor is

Figure 16.

Left: Figure illustrates the rate of ^{125}I-α-BuTx binding to membrane fragments enriched with AcCh receptor. The receptor (0.071 μM in α-BuTx binding sites) was preincubated with 12 ng/ml of peak 4 for 10 minutes. Then ^{125}I-α-BuTx (0.48 μM) was added with no additions (●), with 1 μM Carb (△), and after the receptor was incubated with 1 μM Carb for 10 minutes (▲). The buffer contained 10 mM HEPES-NaOH, 5 mM KCl, 2 mM $MgCl_2$, 4 mM $CaCl_2$, and 250 mM NaCl (pH 7.5).

Right: Same as above except that the 4 mM $CaCl_2$ was omitted from and 1 mM EGTA included into the buffer.


RT (cpm x 10^-3)
10
8
6
4
2
1
2
3
TIME (MIN)


RT (cpm x 10^-3)
10
8
6
4
2
1
2
3
TIME (MIN)

pretreated with peaks B or C, the rate of ^{125}I-α-BuTx binding to the receptor is reduced greatly. Peaks B and C may bind with high affinity to the cholinergic ligand binding site in vivo and similar to α-BuTx, block AcCh binding to the postsynaptic receptor.

Also, Lee et al. (82) observed that the other two toxic components isolated from Bungarus caeruleus venom acted in a manner similar to the presynaptic toxin, β-BuTx. We observe that peaks I and J have low levels of A-type phospholipase activity similar to that of β-BuTx. Further, Kelly and Brown (13) observed that SDS gel electrophoresis of β-BuTx in the presence of reducing agent yields two major polypeptide bands of apparent MW 12,400 and 8,800 daltons, whereas if reducing agent is absent a single higher molecular weight band is observed. They proposed that β-BuTx is composed of two heterologous subunit attached covalently by an interchain disulfide bond. We observe similar results using peaks I or J. These data indicate that peaks I,J, and β-BuTx have several similar biochemical properties.

Bon and Changeux (77) observed that an acidic postsynaptic toxin was present in crude Bungarus caeruleus venom obtained from a private source. This component was eluted from a QAE-Sephadex A-50 anion exchange column when the concentration of NaCl was 125 mM and accounted for at least 35% of the total venom toxicity. Since we did not observed substantial toxicity in peaks 3,4, or 5, we conclude that this component is either present, but not toxic,

or absent in our crude Bungarus caeruleus venom obtained from Sigma Chemical Co. or the Miami Serpertarium.

Since Brisson et al. (83) observed that μM quantities of 8-deoxypalmitic acid, Triton X-100, and Emulphogene BC-720 affect the binding of ^{3}H-AcCh to receptor enriched membrane preparations from Torpedo marmorata electroplax, as well as the depolarization of the Electrophorus electroplaque produced by carbamylcholine, they proposed that any agent which perturbs the lipid environment of the receptor may affect the binding of agonists to the receptor, and in turn the regulation of the ion translocation device. Since we observed that when membrane preparations enriched with AcCh receptor were treated with peak 4, which has high phospholipase A activity, the receptor binding affinity for Carb added simultaneously was increased,we propose that the small quantities of lysophospholipids and/or fatty acids released by exposure of the membrane preparations to exogenous A-type phospholipases affects the receptor conformation. Since Weber et al.(26) observed that Torpedo membranes "age" with time and display increasing affinity for carbamylcholine added simultaneously, and we detect low levels of endogenous A-type phospholipase activity in the Torpedo membranes routinely prepared here (W. Wu, T. Moody, and M.A. Raftery, unpublished), it is quite possible that the activity of endogenous A-type phospholipases contributes to this "aging" process.

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