STUDIES ON LIGAND BINDING TO THE HISTRIONICOTOXIN AND THE AGONIST BINDING SITES OF MEMBRANE BOUND ACETYLCHOLINE RECEPTOR FROM *TORPEDO CALIFORNICA*

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CHAPTER I

ABSTRACT

The binding of various alkylguanidines to the histrionicotoxin site of the membrane bound acetylcholine receptor of Torpedo californica was studied. These results, along with data obtained on the binding of several histrionicotoxin derivatives, were used to formulate a model of ligand to binding to the receptor's histrionicotoxin site.

An azido derivative of histrionicotoxin was used in an attempt to photoaffinity label the histrionicotoxin site. The effect of the agonist carbamylcholine on incorporation of this label was investigated.

CHAPTER II

ABSTRACT

Proton magnetic resonance was used to monitor binding of choline, a known partial agonist, to acetylcholine receptor-enriched membrane preparations from Torpedo californica electroplax. The interaction between choline and receptor led to a broadening of the resonance of the choline methyl groups and this effect was reversed by α-bungarotoxin, a quasi-irreversible antagonist of the acetylcholine receptor. From the concentration dependence of line broadening the equilibrium dissociation constant for choline was obtained (K_d = 190 ± 65 μM). The temperature dependence of the parameters observed in the choline titrations gave an enthalpy of binding $\Delta H < 1.5$ kcal/mol and allowed estimates for the dissociation rate constant of the receptor-choline complex ($k_{diss} > 1.6 \times 10^3$ S^1) and the respective activation energy, E_a (k_{diss}) ≈ 5.5 kcal/mol. The association of other ligands with the membrane-bound receptor could also be studied by observing effects of varying concentrations of such ligands on the choline methyl group linewidth at a constant choline concentration.

CHAPTER III

ABSTRACT

Membrane fragments from the electric organ of Torpedo californica were purified so that the only protein components remaining were the four homologous acetylcholine receptor subunits of 40,000, 50,000, 60,000 and 65,000 daltons. The change in agonist affinity induced by carbamylcholine and the rate of this change, especially in the presence of local anesthetics, were studied and compared to the corresponding properties of membranes containing additional protein components. The results were consistent with the notion that desensitization, as defined by conversion to a state of higher ligand affinity of the acetylcholine receptor, and modulation of the kinetics of this effect by local anesthetics are dependent only on the receptor protein in its native environment.

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Abbreviations used:

AcCh	acetylcholine
AcChR	acetylcholine receptor
α-BuTx	α-bungarotoxin
Carb	carbamylcholine
CBB	Coomassie Brilliant Blue
DEAE	diethylaminoethyl
EDTA	ethylenediaminetetracetic acid
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HTX	histrionicotoxin
H_2-HTX	dihydroisohistrionicotoxin
H_8-HTX	octahydrohistrionicotoxin
H_{10}-HTX	decahydrohistrionicotoxin
H_{12}-HTX	perhydrohistrionicotoxin
HTX-amide	Rel-(6S,7S,8S)-8-(7-butyl-1-azospiro[5.5]undecane-2-one) formate
2R-dehydroxy-HTX	Rel-(2R,6S)-Δ^7-7-butyl-2-pentyl-1-azospiro[5.5]undecene
2S-dehydroxy-HTX	Rel-(2S,6S)-Δ^7-7-butyl-2-pentyl-1-azospiro[5.5]undecene
HTX-H	Rel-(6S,7S,8S)-7-butyl-8-hydroxy-1-azospiro[5.5]undecane
HTX-OH	Rel-(2R,6S,7S,8S)-7-butyl-8-hydroxy-2-(5-hydroxypentyl)-1-azospiro[5.5]-undecane

$HTX-N_3$	Rel-(2R,6S,7S,8S)-7-butyl-8-hydroxy-2-[5-(2-nitro-4-Azidophenoxy)pentyl]-1-Azospiro[5.5]undecane
NMR	nuclear magnetic resonance
SDS	sodium dodecylsulfate
Torpedo Ringer's	250 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$ 4 mM $CaCl_2$, buffered by 20 mM Hepes to pH 7.4

INTRODUCTION

Early studies in microanatomy (Ramon y Cajal, 1894, Auerbach, 1898) demonstrated that there is no direct contact between the nerve ending and the muscle to which the nerve carries information. This gap, or synaptic space (Foster and Sherrington, 1897), implies that the electrical depolarization propagated along the nerve is not directly transferred to the muscle as an electrical impulse, per se, but rather that a separate mechanism exists to bridge the gap, which is 200-500 Å wide. After several decades of research by biochemists and electrophysiologists, the transmission of the nerve impulse across the synapse is known to be mediated chemically. The depolarization of the nerve terminal by a nerve impulse induces the release of acetylcholine, which diffuses across the gap and binds to the acetylcholine receptor protein (AcChR). This binding causes the opening of ion channels in the postsynaptic membrane. The resulting influx of sodium ions depolarizes the postsynaptic membrane and generates an end plate potential, which leads to events culminating in contraction of the muscle.

Depolarization of the presynaptic membrane results in an influx of calcium ions. These calcium ions cause the fusion of many presynaptic vesicles with the presynaptic membrane (Rahamimoff, et al., 1975). These vesicles each contain 10^3-10^5 molecules of acetylcholine and this acetylcholine is released into the synaptic space when the vesicles fuse with the membrane. In the absence of a nerve impulse presynaptic

vesicles occasionally and randomly fuse with the postsynaptic membrane, which results in miniature end plate potentials (MEPP) of about 0.5 mV. These are far below the firing threshold of the muscle (Katz, 1971) but are detectable electrophysiologically and have been an important source of information in investigations of the synapse (Stevens, 1975). A nerve impulse results in the fusion of a large number of vesicles with the membrane and sufficient acetylcholine is released to trigger a full end plate potential in the post synaptic membrane. This end plate potential is sufficiently large to trigger an action potential which propagates down the muscle fiber. The synapse is subsequently cleared of acetylcholine by the acetylcholinesterase, which hydrolyses acetylcholine into acetate and choline.

The key event on the postsynaptic side is in converting the chemical signal back into an electrical one. The binding of acetylcholine to the AcChR must cause an increase in the postsynaptic membrane permeability to sodium ions, which probably pass through an ion channel. The structure of this channel and the mechanism by which the binding of acetylcholine opens the channel are just beginning to be understood.

The discovery that α-Bungarotoxin binds specifically and essentially irreversibly to the AcChR, blocking AcCh binding (Lee and Chang, 1966; Lee, et al., 1967), was the breakthrough that allowed isolation of the AcChR. The toxin is a small peptide which was isolated from the venom of the banded krait Bungarus multicinctus. Well known methods of protein chemistry

allowed labeling of the α-BuTx with ^{125}I or a fluorophore (Barnard, et al., 1971; Berg, et al., 1972; Barrantes, et al., 1975) and demonstrated that the AcChR is located exclusively on the postsynaptic side of the synapse. The membrane is highly folded in this region and the AcChR was shown to be concentrated at the crests of the folds at densities of 2 - 2.5 x $10^4/\mu m^2$ (Barnard, et al., 1975). The cholinesterase is evenly distributed along the folds (Salpeter, et al., 1972).

Isolation of the AcChR was also simplified with the discovery that the electric organs of Torpedo and Electrophorus are rich sources of the protein. These electric organs are modified muscle tissue with large numbers of synapses (Bennett, 1970). The organs constitute a substantial fraction of the total weight of the fish; a 15 Kg Torpedo has approximately 3 Kg of electric organs, one located in each of its two lobes. On a cellular level, the organs are composed of stacks of large cells known as electroplaques. The ventral face of an electroplaque is heavily innervated, with up to fifty percent of the membrane area covered by synapses in Torpedo and perhaps two percent of the membrane in the case of Electrophorus (Bourgeois, et al., 1972). Typically, the higher density of synapses in Torpedo makes them the source of choice for isolating AcChR, while the larger size of electroplaques from Electrophorus favors them as a source for electrophsiological studies with intact cells. The Electophorus electroplaques are large enough to allow isolation and mounting of a single cell between extra-

cellular electrodes and thus the effects of bath applied drugs may be investigated (Schoeffeniels and Nachmansohn, 1957).

Historically the AcChR was first isolated as a detergent solubilized complex with radioactively labeled α-BuTx (Miledi, et al., 1971; Raftery, et al., 1972; Clark, et al., 1972; Eldefrawi and Eldefrawi, 1972). The protocols established in these studies soon led to isolation of the solubilized free AcChR from several species of Torpedo (Raftery, et al., 1973; Schmidt and Raftery, 1973; Eldefrawi and Eldefrawi, 1975; Karlin and Coburn, 1973; Ong and Brady, 1974; Weill, et al., 1974), Narcine (Schmidt and Raftery, 1972), and Electrophorus (Biesecker, 1973; Klett, et al., 1973; Chang, 1974; Lindstrom and Patrick, 1974; Meunier, et al., 1974) using affinity chromatography. Most of these early isolations reported the presence of a protein of 40,000 daltons molecular weight, although there was little agreement beyond this as to the subunit composition of the AcChR. Proteolysis appears to have been the major reason for these differences (Vandlen, et al., 1976) and it has now been established that the AcChR from Torpedo is composed of subunits of molecular weights 40,000, 50,000, 60,000, and 65,000 daltons (Raftery, et al., 1974; Weill, et al., 1974; Vandlen, et al., 1976), with a respective subunit stoichiometry of 2:1:1:1 as determined by N-terminal amino acid analysis (Strader, et al., 1980). This stoichiometry leads to a molecular weight of 255,000 daltons, which compares well with the values of 250,000 daltons determined for Torpedo using sedimentation velocity

centrifugation corrected for bound detergent using D_2O (Reynolds and Karlin, 1978), 270,000 daltons using membrane osmometry (Martinez-Carrion, et al., 1975), and 270,000 daltons calculated by SDS electrophoresis of cross-linked AcChR (Biesecker, 1973; Hucho, et al., 1978). Other sedimentation determinations have reported molecular weights of between 250,000 and 330,000 daltons, but these are complicated by the difficulty in determining the amount of detergent bound to the protein (Raftery, et al., 1972; Edelstein, et al., 1975).

The detergent solubilized AcChR exists as both monomer and dimer with 9S and 13.7S sedimentation coefficients (Raftery, et al., 1972). Both have similar ligand binding properties (Raftery, et al., 1972; Sugiyama and Changeux, 1975; Gibson, et al., 1976). The dimer is formed by a disulfide bond between the 65,000 dalton subunits of two monomers (Chang and Bock, 1977; Hamilton, et al., 1977; Witzemann and Raftery, 1978a; Hucho, et al., 1978).

The unusually high concentration of AcChR in the post-synaptic membrane clefts allows isolation of almost pure AcChR in its native membrane environment. Homogenization of the Torpedo electroplaques results in membrane fragments, a substantial percentage of which are derived from the synaptic clefts. Fragments from the synaptic clefts are of higher density than extra-synaptic cleft fragments due to the densely packed AcChR they contain. This allows isolation of the membrane-bound AcChR by sucrose density centrifugation (Cohen,

et al., 1972; Duguid and Raftery, 1973). Subsequent removal of the extrinsic membrane proteins using alkali (Steck and Yu, 1973) results in preparations of AcChR bound to its native membrane substantially free of contaminating proteins (Neubig, et al., 1979; Elliott, et al., 1980). These AcChR enriched membrane fragments have been valuable in investigating ligand binding and *in vitro* flux of ions through the AcChR ion channels.

These membrane preparations have also been used in structural studies of the AcChR. Electron microscopy of the preparations demonstrated large arrays of densely packed rosettes, each about 90 Å in diameter (Nickel and Potter, 1973; Cartaud, et al., 1973; Raftery, et al., 1974), which were positively identified as AcChR (Klymkowsky and Stroud, 1979). Low angle X-ray studies of the *Torpedo* membrane fragments demonstrated that the AcChR spanned the membrane (Ross, et al., 1977), a fact which was recently confirmed by using anti-receptor antibodies on sealed membrane vesicles (Strader, et al., 1979). Lactoperoxidase catalysed ^{125}I labeling of AcChR enriched membrane fragments (Hartig and Raftery, 1977) has shown that at least the 40,000, 50,000, and 60,000 dalton subunits are exposed to the solvent.

The membrane-bound AcChR preparations have the same subunits as the affinity chromatography purified receptor in addition to bands of 43,000 and 90,000 daltons and small amounts of other proteins. These two proteins are removed by the alkali treatment

and do not serve any known AcChR function (Elliott, et al., 1980; Neubig, et al., 1979; Wu and Raftery, 1980).

The ligand binding properties of the membrane-bound AcChR are considerably different than those of the detergent solubilized preparations. The binding of [^{125}I]-α-BuTx to the membrane-bound AcChR of Torpedo has been shown to follow kinetics which can be described by a simple biomolecular mechanism (Weber and Changeux, 1974; Colquhoun and Rang, 1976; Weiland, et al., 1976); Blanchard, et al., 1979a). This indicates one homogeneous class of toxin binding sites. α-BuTx binding to the Triton X-100 solubilized purified AcChR is more complicated, as biphasic kinetics were seen (Blanchard, et al., 1979a). This implied either two classes of toxin sites or negative cooperativity (Blanchard, et al., 1979a). The rate of α-BuTx binding to solubilized AcChR was also much faster.

Both solubilized and membrane-bound AcChR preparations bind cholinergic ligands, though as with α-BuTx, the two forms have significantly different properties. This difference is especially noticeable in the phenomenon known as desensitization.

Densensitization is the term coined for the observation that prolonged exposure of the in vivo neuromuscular junction to agonists, which are defined as compounds that induce a depolarization of the postsynaptic membrane, decreases the effectiveness of the agonists in eliciting an electrical response (Katz and Thesleff, 1957). This observation can be explained by a two state model, in which an AcChR state with

low ligand affinity slowly converts to one of high agonist affinity due to the influence of agonist (Katz and Thesleff, 1957). The in vivo agonist-induced affinity state change also results in increased affinity for some antagonists (Rang and Ritter, 1969; 1970b) and therefore the high affinity state is not ligand specific. The affinity state change has also been observed in vitro using preparations of membrane-bound AcChR (Weber, et al., 1975; Colquhoun and Rang, 1976; Weiland, et al., 1976; Lee, et al., 1977). A quantitative explanation of in vitro carbamylcholine binding to AcChR requires a model more complex than the two state one of Katz and Thesleff (Quast, et al., 1978b, Dunn, et al., 1980). Triton X-100 solubilized AcChR does not undergo reversible affinity state changes upon exposure to agonist (Vandlen, et al., 1976) possibly indicating that the membrane is important in forming or maintaining receptor conformation.

Attempts have been made to develop kinetic models of agonist binding to membrane bound AcChR using fluorescent probes in conjunction with stopped-flow spectroscopy. The change in intrinsic fluorescence of the AcChR upon ligand binding is quite small, but it has been used to observe a slow conformation change upon addition of agonist said to represent desensitization (Bonner, et al., 1976; Barrantes, 1976). Extrinsic fluorescent probes result in large signals and hence better ability to observe subtle changes in conformation, but the hydrophobic nature of these probes leads to complications when they unavoidably partition

to some extent into the membrane. Perturbations induced by the probe itself are also a problem. Quinacrine was used as a membrane fragment probe and demonstrated two processes upon addition of agonist; one which was thought to represent receptor activation (Grünhagen, et al., 1977b) and a slower one which was attributed to desensitization (Grünhagen, 1976; Grünhagen, et al., 1977a). However, quinacrine is now known to act as a local anesthetic and these kinetic data therefore are suspect.

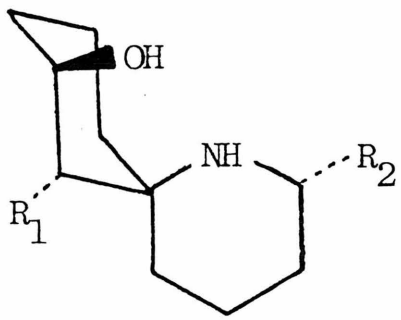
Ethidium bromide has also been used to study agonist binding (Schimerlik, et al., 1979a,b; Quast, et al., 1978a). It does not act as a local anesthetic and data collected using it could best be explained using a sequential binding model (Quast, et al., 1978a; Quast, et al., 1979). Fluorescent acetylcholine analogs have also been used (Jürss, et al., 1979; Heidmann, et al., 1978), as has a method of covalently attaching a fluorescent label near the agonist site (Dunn, et al., 1980).

Local anesthetics are known to block the normal *in vivo* response of the AcChR to agonists. On the basis of electrophysiological evidence it has been proposed that local anesthetics bind to the open channel form of the AcChR, decreasing conductance (Ruff, 1976; Neher and Sakman, 1976). Local anesthetics also increase the rate of desensitization *in vivo* (Magazanik and Vyskocil, 1973; Magazanik, 1976). *In vitro* studies with local anesthetics have demonstrated that they are weakly competitive with α-BuTx (Weber and Changeux, 1974;

Weiland, et al., 1977; Blanchard, et al., 1979b) and with perhydrohistrionicotoxin (Blanchard, et al., 1979b). Local anesthetics also affect the rate of *in vitro* agonists induced desensitization in the membrane-bound AcChR with varying magnitudes. The magnitudes approximately correlate with the binding of a given local anesthetic with the high or the low affinity forms of the AcChR (Blanchard, et al., 1979b). The nature of the local anesthetic binding site is not known, so it is difficult to do more than speculate about the mechanism of local anesthetic effects on the rate of desensitization. Procaine amide azide, a local anesthetic analog, was used as a photoaffinity label of the membrane-bound AcChR (Blanchard and Raftery, 1979) and results obtained with this analog are discussed below.

The histrionicotoxins are another class of ligands that bind to the AcChR. Histrionicotoxins were first isolated from methanolic extracts of the skin of the South American "poison arrow" frog, *Dendrobates histrionicus* (Daly, et al., 1971). The crude venom from these frogs is a mixture of alkaloids, one class of which is the histrionicotoxins, typified by an eleven carbon atom [5.5] azaspiro structure substituted in two positions with a 5 carbon atom and a 4 carbon atom side chain. The various histrionicotoxins differ in the degree of saturation of these side chains (Fig. I) (Tokuyama, et al., 1974) with little difference in biological activity between them (Albuquerque, et al., 1973a,b), The toxins are well

Figure 1. The Histrionicotoxins



HTX: R_1 = -CH=CH-C≡CH, R_2 = $-CH_2CH=CH-C\equiv CH$

H_2-HTX: R_1 = -CH=CH-C≡CH, R_2 = $-CH_2CH_2CH=C=CH_2$

H_8-HTX: R_1 = $-CH_2CH_2CH=CH_2$, R_2 = $-CH_2CH_2CH_2CH=CH_2$

H_{12}-HTX: R_1 = $-C_4H_9$, R_2 = $-C_5H_{11}$

characterized chemically (Daly, et al., 1971; Tokuyama, et al., 1974) and the X-ray structures of the two major forms present in the venom have been reported (Daly, et al., 1971; Karle, 1973). Both H_{12}-HTX (Corey, et al., 1975a) and H_{10}-HTX (Fukuyama, et al., 1975) have been synthesized in the laboratory.

The first electrophysiological studies with HTX (Albuquerque, et al., 1973a,b) found that it did not cause depolarization of the postsynaptic membrane, rather the toxin was found to block depolarization induced by application of AcCh and also that induced by indirect electrical stimulation. This blockade was reversible. Further studies found that the H_{12}-HTX concentrations necessary for half-maximal blockade of the rat muscle endplate potentials and extrajunctional AcCh sensitivity were 8.4 μM and 2.4 μM respectively (Dolly, et al., 1977). H_{12}-HTX blocks α-BuTx binding only at concentrations more than a hundred-fold higher than these. An elaborate body of further electrophysiological data has been accumulated (Albuquerque, et al., 1975; Lapa, et al., 1975).

The effects seen with HTX electrophysiologically are reminiscent of those seen with certain local anesthetics and it has been proposed (Kato and Changeux, 1976) that HTX and these local anesthetics have the same mode of action, namely that they increase the rate of agonist induced desensitization without causing desensitization in and of themselves (Magazanik and Vyskocil, 1973). It has also been proposed (Albuquerque, et al., 1973a,b) that HTX interacts with the open-channel form

of the AcChR, blocking the ion channel. This last hypothesis is tantalizing as it implies that HTX is a possible probe of the ion channel. As no other ion channel probe is known, HTX has been the subject of research in several laboratories (Eldefrawi, et al., 1975; Elliott and Raftery, 1977; Elliott and Raftery, 1979). It has been found that Carb causes a slight increase in the affinity of the membrane-bound AcChR for HTX (Elliott and Raftery, 1977) and HTX increased AcChR affinity for AcCh (Kato and Changeux, 1976). Most importantly, in view of the above two hypotheses, HTX did not increase the rate of Carb induced desensitization of membrane-bound AcChR (Elliott, et al., 1979). Solubilization of the AcChR destroyed HTX binding activity, although this activity could be regained from cholate extracts of AcChR enriched membrane fragments if the detergent concentration was diluted below the critical micelle concentration (Elliott and Raftery, 1979).

The ultimate biological function of the AcChR is to allow a controlled influx of sodium ions through the postsynaptic membrane, hence reproducing this function in vitro with a system of well defined protein and lipid components is a necessary step toward developing a full understanding of the AcChR mechanism.

A large fraction of the AcChR enriched membrane fragments isolated from the sucrose density gradients are intact vesicles. These vesicles can be loaded with $^{22}Na^{+}$ and have sufficient functional integrity that agonist induced $^{22}Na^{+}$ efflux through

the ion channel can be monitored through use of a filter assay (Kasai and Changeux, 1971; Michaelson and Raftery, 1974; Hazelbauer and Changeux, 1974; Popot, et al., 1976; Hess and Andrews, 1977; Miller, et al., 1978; Moore, et al., 1979). The method is too slow to be useful for kinetic studies, but does show the time dependent inactivation by agonists thought to be *in vitro* desensitization (Miller, et al., 1978). The amplitude of the flux is dependent on the agonist concentration (Bernhardt and Neumann, 1978; Miller, et al., 1978; Neubig, et al., 1979; Moore, et al., 1979). The flux is blocked by both α-BuTx and HTX, as expected from *in vivo* studies with these ligands. Elegant flux measurements of alkali treated membrane-bound AcChR in which known fraction of the AcChR had been blocked by α-BuTx demonstrated that the four subunits contained in these membrane fragments and not some minor component are responsible for ion flux (Moore, et al., 1979).

Improved technology has allowed measurement of *in vitro* ion flux on a time scale short enough to allow comparison with *in vivo* data. Thallium ion influx into vesicles loaded with a fluorescent molecule results in a quenching of the dye and allows observation of the agonist induced Tl^+ flux with only a 2.5×10^{-3} sec dead time in the stopped-flow spectrometer (Moore and Raftery, 1980). The single channel flux rate is comparable to the rate *in vivo*. Thus *in vitro* flux for preparations of defined protein composition has been demonstrated and the specific lipid composition necessary, if any, is the

remaining unknown. Modification of the lipid composition requires solubilization of the membrane fragments and here also a great deal of progress has been made.

Early attempts at reconstituting solubilized AcChR had to make use of AcChR solubilized in non-ionic detergents and then purified by affinity chromatography. It is now known that the AcChR is sensitive to minute amounts of bound detergent and also has important sulfhydryl groups which affect activity, thus it is not surprising that early reports of success in reconstituting agonist induced ion flux in AcChR containing vesicles (Michaelson and Raftery, 1974) proved difficult to reproduce consistently (Karlin, et al., 1975; McNamee, et al., 1975). When membrane fragments containing AcChR were solubilized with sodium cholate and then reformed into vesicles without further purification, preparations consistently capable of ion flux could be produced (Hazelbauer and Changeux, 1974). Functional preparations could also be obtained by adding large amounts of exogenous lipids to cholate solubilized membrane fragments (Epstein and Racker, 1978). The advent of alkali treatment of membrane fragments allowed production of cholate extracts containing essentially pure AcChR and subsequent reconstitution of these extracts into functional vesicles (Wu and Raftery, 1979; Changeux, et al., 1979; Wu and Raftery, 1980). These vesicles were sealed and capable of agonist induced ion flux which could be blocked by α-BuTx or HTX (Wu and Raftery, 1980).

It is now generally accepted that the AcChR of Torpedo is composed of three (Neubig, et al., 1979) or four subunits (Raftery, et al., 1979; Weill, et al., 1974; Raftery, et al., 1975 ; Chang and Bock, 1977; Lindstrom, et al., 1978; Vandlen, et al., 1979; Strader, et al., 1980). The subunits are of molecular weights 40,000, 50,000, 60,000 and 65,000 daltons and are present in a stoichiometry of 2:1:1:1 (Strader, et al., 1980a). The locations of the various binding sites on these subunits are not completely known, nor is the composition of the ion channel.

Bromoacetylcholine (Chang, et al., 1977; Damle, et al., 1978; Moore and Raftery, 1979a) and 4-(N-maleimido)benzyltrimethylammonium iodide (Weill, et al., 1974) were used to covalently label the AcCh binding site, which is known to possess a disulfide bond which can be reduced and labeled with sulfhydryl reagents like the above. The labeling experiments found that the labels were incorporated into the 40,000 dalton subunit. Cholinergic analog photoaffinity reagents have been synthesized and used with some success. Bis(3-azidopyridinium)-1,10-decane perchlorate labeled the 40,000 and 50,000 dalton subunits of membrane-bound AcChR, while it labeled the 40,000 and 60,000 dalton subunits of Triton X-100 solubilized AcChR purified by affinity chromatography (Witzemann and Raftery, 1977). Bis(azido)ethidium chloride labeled the 40,000 dalton component of AcChR enriched membrane fragments and this labeling was partially blocked by α-BuTx

(Witzemann and Raftery, 1978b). Incubation of the membrane fragments with agonist prior to labeling increased the amount of bis(Azido)ethidium incorporated into the 40,000 and also the 50,000 and 65,000 dalton subunits. Solubilized AcChR was labeled using 4-azido-2-nitrobenzyltrimethylammonium fluorborate (Hucho, et al., 1978). While all four subunits incorporated label only the labeling of the 40,000 dalton subunits was prevented by preincubation with α-BuTx. The conclusion of these studies is that the 40,000 dalton subunit contains the agonist site and that either the other subunits are in such close proximity to this site that they are labeled by diffusion of the photoactivated analog from the site on the 40,000 dalton subunit or that there are secondary sites for the analogs on the other subunits. These secondary sites may have evolved from a single original site given the discovery that the four subunits are homologous (Raftery, et al., 1980).

α-BuTx-azide has also been synthesized and found to label the 40,000 and 65,000 dalton subunits (Witzemann, et al., 1979). This indicates that the 65,000 dalton subunit was exposed to the aqueous phase in addition to the 40,000, 50,000 and 60,000 subunits as demonstrated earlier by lactoperoxidase catalyzed iodination (Hartig and Raftery, 1977).

The local anesthetic analog procaine amide azide(PAA) was synthesized and labelled principally the 43,000 and 90,000 dalton proteins of Torpedo membrane fragments, with smaller amounts of label incorporated into the AcChR subunits (Blanchard and

Raftery, 1979). Alkali treatment of the membrane fragments removed the 43,000 dalton and most of the 90,000 dalton labeling allowing observation of PAA binding to the 40,000, 60,000 and 65,000 dalton subunits (Blanchard, 1980). Carb preincubation protected the 40,000 dalton subunit from labeling so that the PAA binding observed on this subunit may have been due to binding of the analog to the agonist site. Non-specific binding and presence of substantial analog affinity at a site not biologically relevant to the analog are major problems with photo affinity reagents, especially those which are hydrophobic.

Substantial interest was shown by the medical community (Check, 1978) in the finding that symptoms similar to myasthenia gravis could be induced in rats by injecting them with any of the four subunits of AcChR isolated from Torpedo (Lindstrom, et al., 1978). The conclusion that experimental autoimmune myasthenia gravis (EAMG) is caused by antibodies to the AcChR and subsequent degradation of those AcChR-antibody complexes led to a substantial revision of thinking on the causes of myasthenia gravis in humans.

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CHAPTER I

Ligand Binding Studies Involving the HTX Binding Site of Membrane Bound Acetylcholine Receptor

INTRODUCTION

The histrionicotoxins were first isolated from the South American "poison arrow" frog Dendrobates histrionicus (Daly, et al., 1971). The compounds are alkaloids which differ in the degree of saturation of their side chains but retain the same basic Azaspiro structure. The natural poison extracted from the frog is composed chiefly of HTX and H_2-HTX (Daly, et al., 1971) and minor amounts of more saturated derivatives (Tokuyama, et al., 1974). The fully saturated derivative perhydrohistrionicotoxin, H_{12}-HTX is not found naturally but has been synthesized by catalytic reduction of natural HTX and by several routes of total synthesis (Aratani, et al., 1975; Fukuyama, et al., 1975; Corey, et al., 1975, 1977; Cherpeck, 1980). H_{12}-HTX has been shown to have biological activity similar to HTX (Albuquerque, et al., 1973a). Histrionicotoxins are based on an eleven carbon Azaspiro ring which has several possible conformations. X-ray crystallography of the HCl salt of HTX indicates that the ring is in a chair conformation (Daly, et al., 1971), placing the hydroxyl group in close proximity to the amino group. With this conformation, the molecule is approximately an oblate ellipsoid, with the two hydrophilic groups oriented in one face, leaving the opposite face completely hydrophobic. The ring conformation does not seem dependent on the degree of chain saturation (Daly, et al., 1971; Karle, 1973). The conformation has interesting implications for the structure of the HTX binding site.

Perhydrohistrionicotoxin (Fig. 1, I) is a small molecule which non-competitively blocks the acetylcholine induced depolarization of the postsynapic membrane (Albuquerque, et al., 1973a, 1973b). It has been shown that this block is caused by specific interaction of the toxin molecule with the acetylcholine receptor (Albuquerque, 1973a, 1973b; Eldefrawi, et al., 1975; Elliott, et al., 1979), possibly by interacting with the ion channel, although an alternative hypothesis (Kato and Changeux, 1976) is that HTX greatly accentuates the process of densitization in a manner similar to that proposed for local anesthetics (Magazanik and Vyskocil, 1973). Because this molecule is one of the few which may interact with the ion channel of the AcChR, it has been the subject of intensive investigation (Kato, et al., 1974; Albuquerque, et al., 1974; Elliott and Raftery, 1979 ; Eldefrawi, et al., 1977; Neubig, et al., 1979).

^{3}H-H_{12}-HTX binds to the membrane bound AcChR present in Torpedo electroplax with a K_d of 0.3 to 0.5 μM (Elliott and Raftery, 1977). The K_d of [^{3}H]-H_{12}-HTX decreases about two-fold when the AcChR is incubated with 10 μM Carb prior to the HTX binding assay (Elliott and Raftery, 1979), which may reflect a conformational change in the AcChR related to desensitization.

Detergent solubilization of the AcChR enriched membranes destroys the ability of the AcChR to bind HTX (Elliott and Raftery, 1977, 1979). This fact, along with the effects of low concentrations of amphipathetic molecules on [^{3}H]-H_{12}-HTX

binding (Elliott, et al., in press), may indicate that the membrane itself constitutes a vital part of the structure of the HTX binding site. Positively charged amphiphiles such as local anesthetics, compete quite strongly with [^{3}H]-H_{12}-HTX for its binding site on the AcChR (Blanchard, et al., 1979b) while neutral amphiphiles are much less effective (Elliott, et al., submitted). Octylguanidine, known to block frog endplate depolarization (Narahashi, 1980) has been found to be particularly effective in competing with [^{3}H]-H_{12}-HTX with an apparent K_d of 1.5 μM. Methylguanidine also blocks endplate depolarization, albeit more weakly than octylguanidine (Narahashi, 1980) and has also been shown to compete with [^{3}H]-H_{12}-HTX with a K_d of 50 mM. The large difference in effectiveness between octyl and methyl guanidines suggested that the hydrophobic elements of the ligand might be important determinants of binding affinity. A series of experiments has been done to determine the binding properties of alkylguanidines to AcChR enriched membrane fragments in an effort to establish a clear relation between hydrophobicity and ligand binding.

I have also investigated the binding properties of several derivatives of HTX. Results of these experiments, together with the alkylguanidine data, lead me to propose a model for binding in which the membrane lipids form the hydrophobic domain of the AcChR HTX binding site.

An azide analog of HTX was the generous gift of Dr. R. Cherpeck and Professor D. Evans. This analog

was tritiated and shown to bind to the HTX site of the AcChR with high affinity. Upon photolysis the analog was incorporated into the 40K , 50K and 65K subunits of the AcChR, though only that binding to the 65 K dalton subunit could be completely blocked by competition with H_8-HTX.

MATERIALS AND METHODS

I. General

H_{12}-HTX and H_8-HTX were the gifts of Professor Y. Kishi. [^{3}H]-H_{12}-HTX was produced by tritiation H_8-HTX which was performed by ICN Pharmaceuticals, Inc. (Irvine, California). Purification and standardization of the [^{3}H]-H_{12}-HTX have been described (J. Elliott, 1979). HTX-OH, HTX-amide and dehydroxy-HTX were gifts from Dr. R. Cherpeck and Professor D. Evans.

Crude Bungarus multicintus venom was obtained from Sigma Chemical Co. This venom was purified via ion exchange chromatography (Clark, et al., 1972). The α-BuTx component of the venom was then iodinated using Na[^{125}I]. The monoiodo derivative was isolated and standardized (Blanchard, et al., 1979a). The [^{125}I]-α-BuTx had biological activity indistinguishable from the unlabeled α-BuTx and had specific activities that ranged from 1-3 Ci/mmole.

Octanol and alkylamines were obtained from Aldrich Chemical Co., methylguanidine was obtained from Sigma Chemical Co. Octylguanidine was the gift of Professor Narahashi.

DE-81 DEAE filter paper discs were obtained from Whatman, Ltd. Scintillation counting was done using a Packard Tricarb Liquid Scintillation Spectrometer or a Beckman LS-233 Liquid Scintillation Spectrometer using Aquasol (New England Nuclear) on 25% v/v Triton X-100, 0.55% Permablend III (Packard) in Toluene. Glass scintillation vials (Wheaton) with sealing

caps were necessary for experiments in which SDS Polyacrylamide gel slices were digested in the vials.

Protein concentrations were determined using the method of Lowry, et al. (1951).

The concentration of [^{125}I]-αBuTX binding sites in the various preparations was assayed using the DEAE disc assay of Schmidt and Raftery (1972).

Fluorescence spectroscopy was done using a Perkin-Elmer MPF-4 fluorometer. All NMR spectra were recorded with a Varian EM-390 NMR spectrometer.

II. AcChR Enriched Membrane Fragments

Approximately 500g of frozen electric organs from T. californica were broken into small pieces and mixed with 500 mL of buffer [10 mM NaPi, 5 mM EDTA, 0.02% NaN_3, 5 mM iodoacetamide, 10 mM PMSF pH = 7.4]. This was ground for 2 minutes in a Waring Blendor, then reground for 4 x 30 seconds at 30,000 rpm in a Virtis 60 homogenizer. Large particles and connective tissue were removed by a 10 minute centrifugation at 5000 rpm in a Sorval GSA rotor. The supernatant was then centrifuged 60 minutes at 30,000 rpm in a Beckman 35 rotor. The pellet was resuspended in 10 mM NaPi (pH 7.4) 1 mM EDTA by using 2 x 30 - second homogenizations with a Virtis 60 homogenizer. Sucrose and NaCl were added to achieve final concentrations of 30% (w/w) and 0.4 M, respectively.

The sample (10 ml) was layered on a discontinuous sucrose gradient with steps of 5 ml 50% w/w, 5 ml 39% w/w, and 12 ml of

30% w/w, all in 0.4 m NaCl, 10 mM Pi (pH = 7.4) 1 mM EDTA. This buffer was used to adjust the final volume of each tube. The loaded tubes were centrifuged for 60 minutes at 45,000 rpm in a VTi 50 rotor using a slow acceleration accessory and Beckman L-5B centrifuge. After centrifugation the membrane fragments were observed to be in three bands. The middle band, consisting of particles with a density of 1.18 g/cc, was aspirated away from the gradients, diluted with buffer and centrifuged for 60 min at 35,000 rpm in a Beckman 35 rotor. The membranes were resuspended in a small amount (∿10 ml) of Torpedo Ringers buffer using a Virtis homogenizer. This preparation is described in detail in Elliott, et al. (1980).

III. Alkali Treatment of AcChR Enriched Membrane Fragments

Alkali treatment of the membrane fragments from the above procedure was adapted from the protocol reported by Steck and Yu (1973) for erythrocyte ghosts. Membrane fragments containing approximately 50 mg of total protein were pelleted, resuspended in distilled water with a Virtis homogenizer and pelleted again. They were then resuspended in 80 ml of cold distilled water using a Virtis homogenizer for 2 x 30 sec. The suspension was then adjusted to pH 11.0 using 0.5% NaOH. This pH was maintained for 30 minutes at 4°, with small additions of NaOH. The membranes were then pelleted by centrifugation for 30 minutes at 30,000 rpm in a Beckman type 35 rotor. A Virtis homogenizer was used to resuspend the pellet in Torpedo Ringer's buffer.

IV. Synthesis of Alkyguanidines

Alkylguanidines were synthesized by reacting S-ethylthiourea with the appropriate alkylamine, according to the procedures of Brand and Brand (1942) as follows:

A. S-ethylthiourea Hydrobromide. Fifteen grams of thiourea were mixed with twenty-five grams of ethylbromide dissolved in 20 ml of dry ethanol. The mixture was refluxed overnight. The solvent was removed using a rotary evaporator and the resulting oil was allowed to crystallize overnight at -20°. The crystals were removed by filtration and subsequently washed with ethanol and dried. The yield was 34 g, which was 94% of the theoretical yield.

B. Alkylguanidines. The desired alkylguanidine derivative may be prepared by reacting S-ethylthiourea with an alkylamine having a side chain of the desired length; $(NH_2)_2C SC_2H_5) + R\text{-}NH_2 \rightarrow (NH_2)_2\text{'-C-NH-R} + C_2H_5SH$. This reaction was accomplished by dissolving .05 moles of alkylamine in either 25 ml of cold 2.0 N NaOH or, in the case of alkylamines with side chains longer than 4 carbon atoms, a mixture of 80% tetrahydrofuran and 20% 10.0 N NaOH. Solid S-ethylthiourea (.05 moles) was slowly added to this mixture with constant stirring. The reactants were stirred at room temperature overnight inside a fume hood. The solvent was then removed with a rotary evaporator and the remaining solid, which is a mixture of NaBr, product, unreacted starting material and small amounts of urea, was triturated with n-butanol. The butanol was decanted away from

the remaining solids and neutralized using ethanol saturated with HCl gas. The HCl salt of the R-guanidine was then recovered by evaporation. Recrystallization could usually be achieved from butanol. Yields were typically about 50% of the theoretical maximum.

C. Dansylguanidine (Fig. 1; VII). Dansylguanidine was synthesized in three steps. First, cystamine was reacted with S-ethylthiourea to form 1,4 bis(guanido) cystamine using the protocol above. This product was dissolved in water and reduced with a two-fold excess of sodium borohydride to yield 2-guanido ethanethiol. Forty mg (330 μ moles) of 2-guanidoethanethiol was dissolved in 20 ml of 0.1 M carbonate buffer (pH 8.3) and was mixed with 80 mg (290 μ moles) of Dansylaziridine (Molecular Probes, Inc.), dissolved in 10 ml of ethanol. This mixture was refluxed for 12 hours, cooled and evaporated to dryness using a rotary evaporator. The product was purified as described in part B of this section. The yield was about 10%. The low yield was due mainly to high handling losses of the small amount of material.

V. Synthesis of HTX-H (Fig. 1, III)

Synthesis of HTX-H was accomplished by reduction of HTX-amide (Fig. 1, IV) with lithium aluminum hydride. A 100 ml round bottom flask and small refluxing condenser were assembled, purged and slightly pressurized with dry argon. Into this apparatus was introduced 19 mg (500 μ moles) of lithium aluminum hydride dissolved in 5 ml of anhydrous diethyl ether. Then 26.2 mg (107 μ moles) of HTX-amide was dissolved in 5 ml of dry diethylether

Figure 1. Histrionicotoxin derivatives. Chemical names for derivatives in these experiments may be found in the abbreviations section.

I) H_{12}-HTX

II) HTX-OH

III) HTX-H

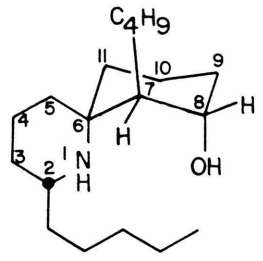
IV) HTX-amide

V) dehydroxy-HTX

VI) alkylguanidine

VII) dansylguanidine

VIII) HTX-N_3



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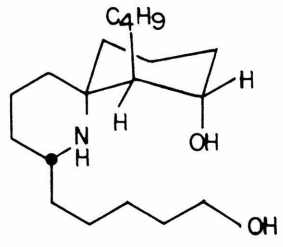

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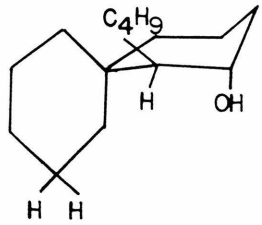
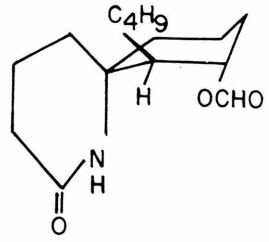


III



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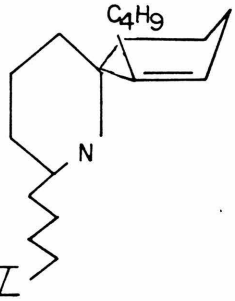
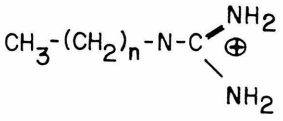
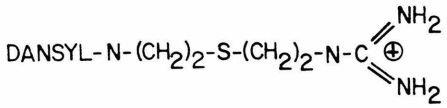


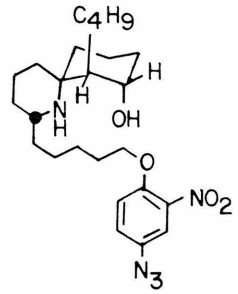
V



VI



VII



VIII

and this was slowly added to the contents of the flask. The reactants were refluxed under dry argon for 8 hours.

The mixture was then cooled in an ice bath and a large excess of $Na_2SO_4 \cdot 10\ H_2O$ was slowly added. The Na_2SO_4 was then filtered away from the reaction mixture and the ether phase evaporated to dryness. Thin layer chromatography on a silica gel plate eluted with chloroform:isopropanol:aqueous ammonium hydroxide (9:2:0.16) showed only one component when stained with iodine. The NMR spectrum was consistent with a compound of structure III in Fig. 1.

VI. Determination of Ligand Binding Constants

Inhibition constants of the various ligands were determined by competition with HTX or with Carb. Membrane fragments were mixed with [^{3}H]-H_{12}-HTX or [^{3}H]-Carb and titrated with the ligand of interest. The mixture was allowed 30 minutes to equilbrate and then the membranes were pelleted using a Beckman Airfuge. The concentration of [^{3}H]-H_{12}-HTX or [^{3}H]-Carb remaining in the supernatant was measured and from this value the fraction of [^{3}H]-H_{12}-HTX or [^{3}H]-Carb bound to the membrane fragments at a given titrant concentration was determined. The amount of non-specific binding to the membrane was determined by saturation of specific sites with H_8-HTX before centrifugation. The centrifugation assay for HTX binding was originally described by Elliott and Raftery (1977). Using the known values for the dissociation constants of [^{3}H]-H_{12}-HTX and [^{3}H]-Carb allowed

correction of the ligand inhibition constants into dissociation constants.

All assays were performed in Torpedo Ringers (250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, buffered to pH 7.4 by 20 mM Hepes) except for the assays of HTX binding versus pH. In these assays the divalent ions were omitted and the pH was maintained by 50 mM sodium citrate for assays between pH 3 and 6.5, 50 mM sodium phosphate for pH values between 7 and 7.5, 50 mM sodium pyrophosphate for pH values between 8 and 9, 50 mM sodium carbonate for pH 9.5 and pH 10, and 50 mM sodium phosphate (tribasic) for pH values above 10. The sodium concentration was maintained at a constant 150 mM by addition of 1 M NaCl.

VII. Determination of Alkylguanidine Partition Coefficients

The relative partition coefficients of long chain alkylguanidines between the membrane fragments and Torpedo Ringers buffer were determined using a fluorimetric assay based on the reaction of ninhydrin with guanidine derivatives to give a fluorescent product (Conn and Davis, 1959).

Membrane fragments were mixed with the alkylguanidine derivative in question to a final ligand concentration of 10^{-4} M. The conditions were chosen so that the assay mixtures were about 2 mM in phospholipid concentration and 2 μM in α-BuTx site concentration. After a 30 minute equilibration period, the membrane fragments were pelleted by centrifugation and the supernatant was assayed for alkylguanidine concentration as

follows: 1 ml of 0.5% aqueous ninhydrin was added to 0.9 ml of water and 0.1 ml of the supernatant sample. The solution was then mixed and 1 ml of 1.0 N KOH was added. After a 15 min incubation the fluorescence of 495 nm was measured using an excitation wavelength of 390 nm. A standard curve for the alkylguanidine being assayed was determined simultaneously. The ratio of the supernatant ligand concentration to the starting concentration was taken as the partition coefficient. The method was not useful for short chain ($\leq$ 4 carbon atoms) alkylguanidines, as the difference between the starting mixture and supernatant was small.

VIII. AcChR Subunit Labeling with HTX-N_3

$^1H_2$-HTX-N_3 (Fig. 1, compd. VIII) was synthesized by Dr. R. Cherpeck (1980) by reacting 4-fluoro,2-nitrophenylazide with HTX-OH (Fig. I, compd. II). This material was tritiated using the Wilzbach method by ICN Pharmaceuticals Inc. (Irvine, California). The crude [^{3}H]-HTX-N_3 (Fig. 1, cmpd. VIII) was purified on a 5 x 100 mm silica gel column eluted with 9:2:0.16 chloroform: isopropanol:aqueous ammonium hydroxide. This and all subsequent purification and assay manipulations were done using only the light from a 25 W red light bulb. A fraction was obtained in which the radioactivity comigrated with $^1H_2$-HTX-N_3 that was used as a standard. This fraction was evaporated to dryness and a small amount was weighed and dissolved in ethanol. The specific activity was 0.3 Ci/mmole.

The binding affinity of [^{1}H]-HTX-N_3 to AcChR enriched membrane fragments was determined by competition with [^{3}H]-HTX using the centrifugation assay described above, while the affinity of the [^{3}H]-HTX-N_3 was measured directly by the centrifugation assay.

The membrane fragments were incubated in the dark for 30 minutes with [^{3}H]-HTX-N_3 at the desired concentration, then placed in a cylindrical quartz cuvette and irradiated 15 minutes with stirring. The light source was a UVSL-25 lamp (Ultraviolet Products , San Gabriel, California) set to the "long" wavelength. The membrane fragments were then pelleted by a 5 minute centrifugation with an Eppendorf 3200 centrifuge. The pellet was resuspended in Torpedo Ringers and pelleted once again. This pellet was dissolved in SDS sample buffer (20% sucrose w/w, 3% w/v SDS, 62.5 mM Tris pH 6.8, 50 mM DTT and 0.1% bromophenol blue).

The sample was applied to a 1 x 10 cm SDS polyacrylamide gel (8.75% acrylamide) and electrophoresed using the method of Laemmli (1970). The gels were subsequently sliced without staining with a Hoeffer Scientific Instruments SL-280 gel slicer. Each slice was placed in a glass scintillation vial (Wheaton Co.) and 0.5 ml of 30% hydrogen peroxide was added. After tightly capping the vials, the samples were incubated six hours at 70°C. The vials were then cooled and 10 ml of scintillation fluid were added . The radioactivity present in the vials was determined using a Beckman LS-233 liquid scintillation spectrometer.

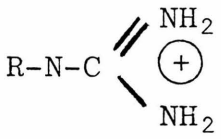
RESULTS

I. Ligand binding to the HTX site

Alkylguanidines with chain lengths from one to eight carbons were found to compete with the binding of [^{3}H]-H_{12}-HTX to the AcChR enriched membrane fragments (Table I). The titrations all resulted in normal Bjerrum-type sigmoidal curves (Figs. 2 - 4). In addition the amount of non-specific [^{3}H]-H_{12}-HTX binding remained constant throughout the range of the titration, regardless of the chain length of the titrant (Figs. 2 - 4). The inhibition constants of the alkylguanidine derivatives were strongly dependent on the alkyl chain length with the longer chains inhibiting more strongly (Table I). The K_ds changed by over 4 orders of magnitude in the range of linear side chains tested. Non-linear alkylguanidines also exhibited specific competition with [^{3}H]-H_{12}-HTX (Fig. 2) with K_ds dependent on the hydrophobicity rather than the total chain length (Table I). Other amphiphiles, such as octylamine and octanol, were tested for their ability to compete with [^{3}H]-H_{12}-HTX; octylamine did compete, although with an affinity more than two orders of magnitude weaker than octylguanidine (Table I). Octanol had no effect on [^{3}H]-H_{12}-HTX binding at concentration up to 0.3 nM, which was the practical limit of the titration due to the limited solubility of the titrant.

The plot of log K_d vs chain length (Fig. 5) of the alkylguanidines suggested that the partition coefficient of the ligand was a critical factor in determining its affinity for the AcChR HTX binding site. The relative partition coefficients between

Table I. Alkylguanidine Dissociation Constants Determined by Competition with ^{3}H-H_{12}-HTX



R	K_d (M)
Methyl	5.0×10^{-2}
Ethyl	4.5×10^{-2}
n-Propyl	1.5×10^{-2}
n-Butyl	1.0×10^{-2}
n-Pentyl	1.8×10^{-3}
neo-Pentyl	1.9×10^{-3}
n-Hexyl	4.0×10^{-4}
Cyclohexyl	1.9×10^{-3}
n-Heptyl	8.0×10^{-5}
n-Octyl	1.5×10^{-6}
Dansyl-N-$(CH_2)_2$-S-$(CH_2)_2$	2.7×10^{-5}
Other ligands:	
n-Octanol	$>> 3 \times 10^{-4}$
n-Octylamine	5×10^{-4}

Table II. Dissociation Constants of Alkylguanidines Determined by Competition with [^{3}H]-Carb

Methylguanidine	3×10^{-3}M
n-Propylguanidine	1.5×10^{-3}M
n-Pentylguanidine	3×10^{-3}M

Table III. Dissociation Constants of HTX Derivatives Determined by Competition with [^{3}H]-H_{12}-HTX

HTX-H	1.2×10^{-5}M
HTX-OH	1.5×10^{-5}M
2R-Dehydroxy-HTX	1.4×10^{-6}M
2S-Dehydroxy-HTX	1.1×10^{-6}M
HTX-N_3	$1\ 5 \times 10^{-6}$M

Figure 2. The competition of methylguanidine with [^{3}H]-H_{12}-HTX binding. AcChR enriched membrane fragments (1.5 μM in α-BuTx sites) were titrated with methylguanidine. The membrane fragments were suspended in Torpedo Ringers buffer with 0.5 μM [^{3}H]-H_{12}-HTX (●-●-●) or 0.5 μM [^{3}H]-H_{12}-HTX + 44 μM H_8-HTX (▲-▲-▲). After 30 minutes of incubation at room temperature, the membrane fragments were pelleted and the amound of radioactivity remaining in the supernatant was determined.

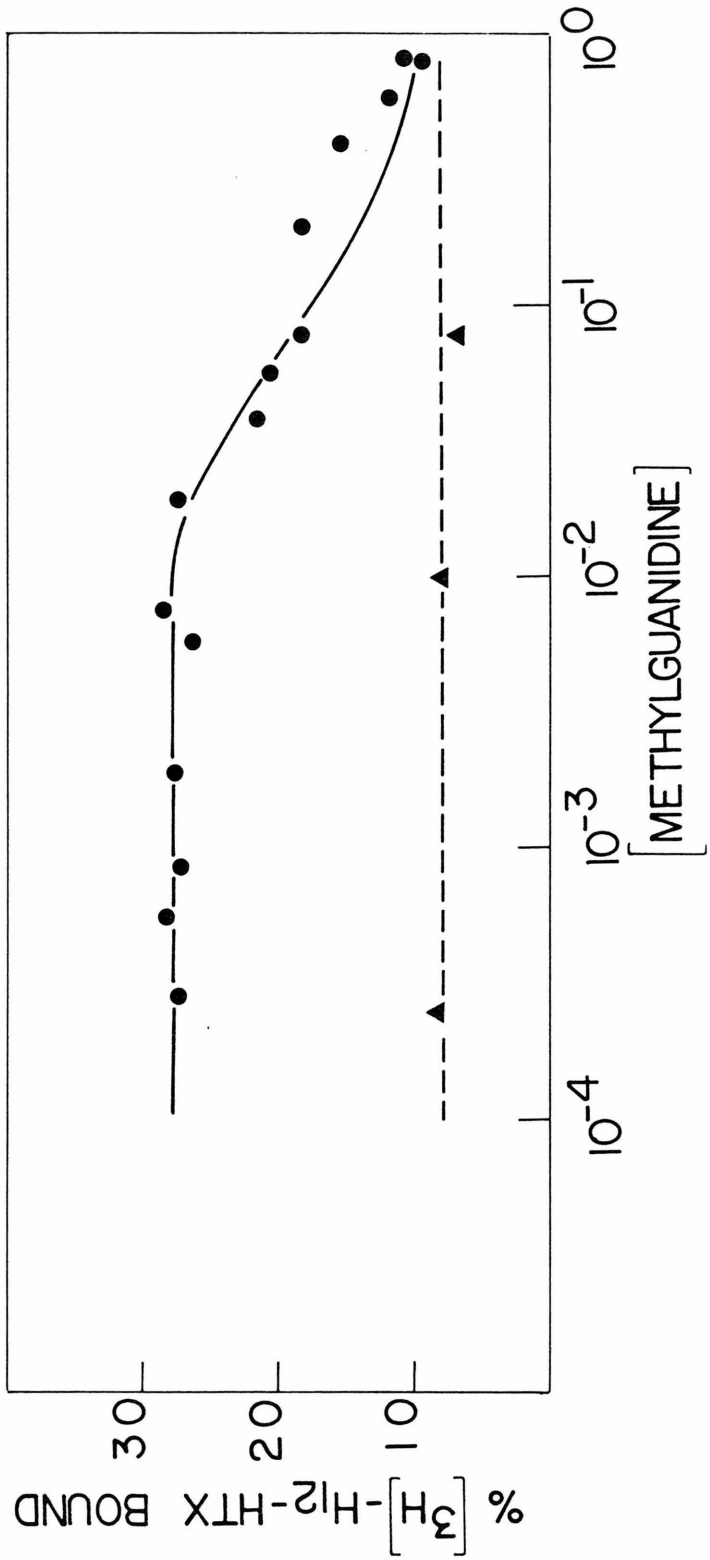

% [3H]-H12-HTX BOUND
30
20
10
10^-4
10^-3
10^-2
10^-1
10^0
[METHYLGUANIDINE]

Figure 3. The competition of n-Pentylguanidine with [^{3}H]-H_{12}-HTX binding. AcChR enriched membrant fragments (1.5 μm in α-BuTx sites) were titrated with n-pentylguanidine. The membrane fragments were suspended in Torpedo Ringer's buffer with 0.5 μM [^{3}H]-H_{12}-HTX (●-●-●) or 0.5 μM [^{3}H]-H_{12}-HTX plus 44 μM H_8-HTX (▲-▲-▲). After 30 minutes of incubation at room temperature, the membrane fragments were pelleted and the amount of radioactivity remaining in the supernatant was determined.

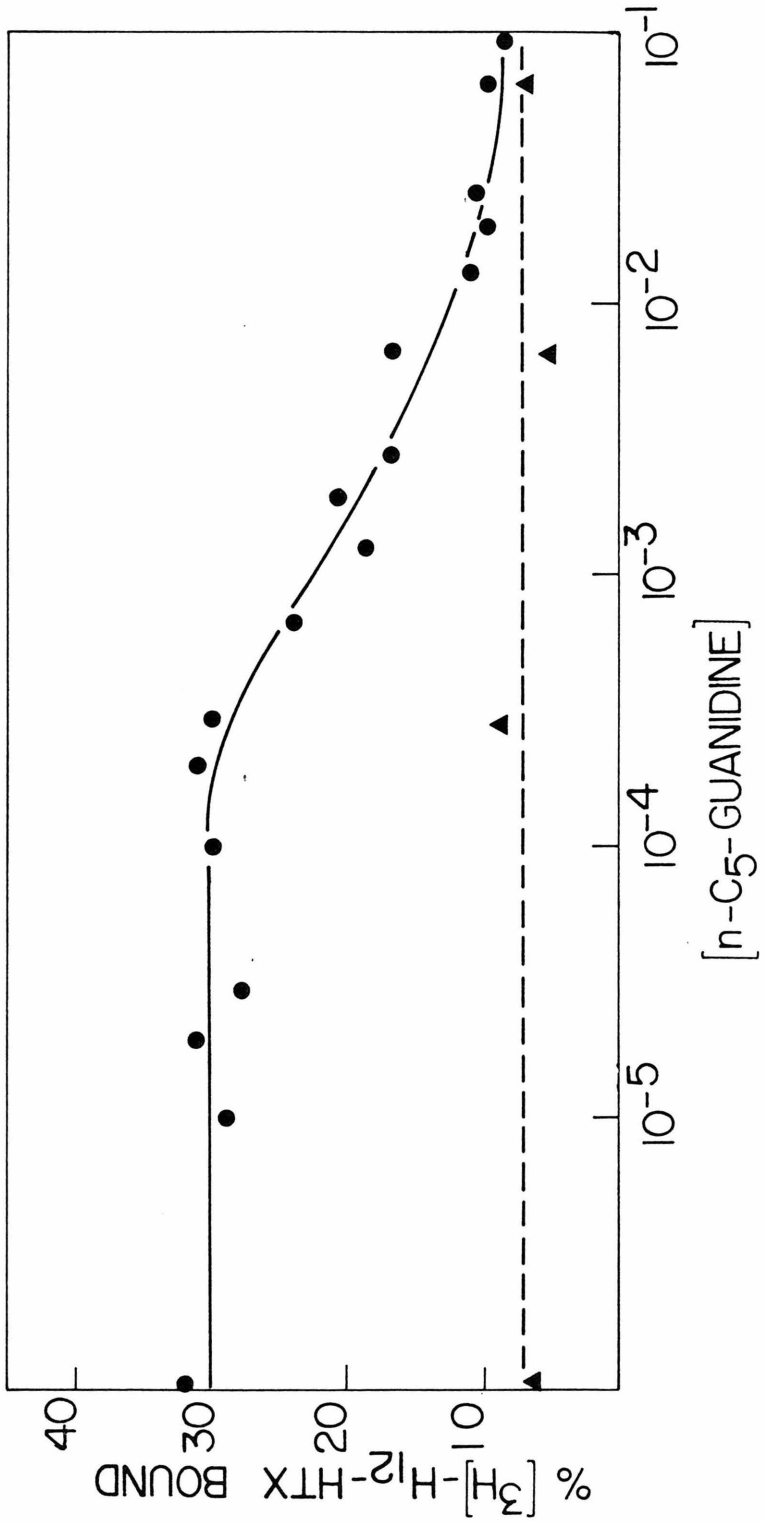

% [3H]-H12-HTX BOUND
40
30
20
10
10-5
10-4
10-3
10-2
10-1
[n-C5-GUANIDINE]

Figure 4. The competition of n-Heptylguanidine with [^{3}H]-H_{12}-HTX binding. AcChR enriched membrane fragments (1.5 μM in α-BuTx sites) were titrated with n-heptylguanidine. The membrane fragments were suspended in Torpedo Ringer's buffer with 0.5 μM [^{3}H]-H_{12}-HTX (●-●-●) or 0.5 μM [^{3}H]-H_{12}-HTX plus 44 μM H_8-HTX (▲-▲-▲). After 30 minutes of incubation at room temperature, the membrane fragments were pelleted and the amount of radioactivity remaining in the supernatant was determined.

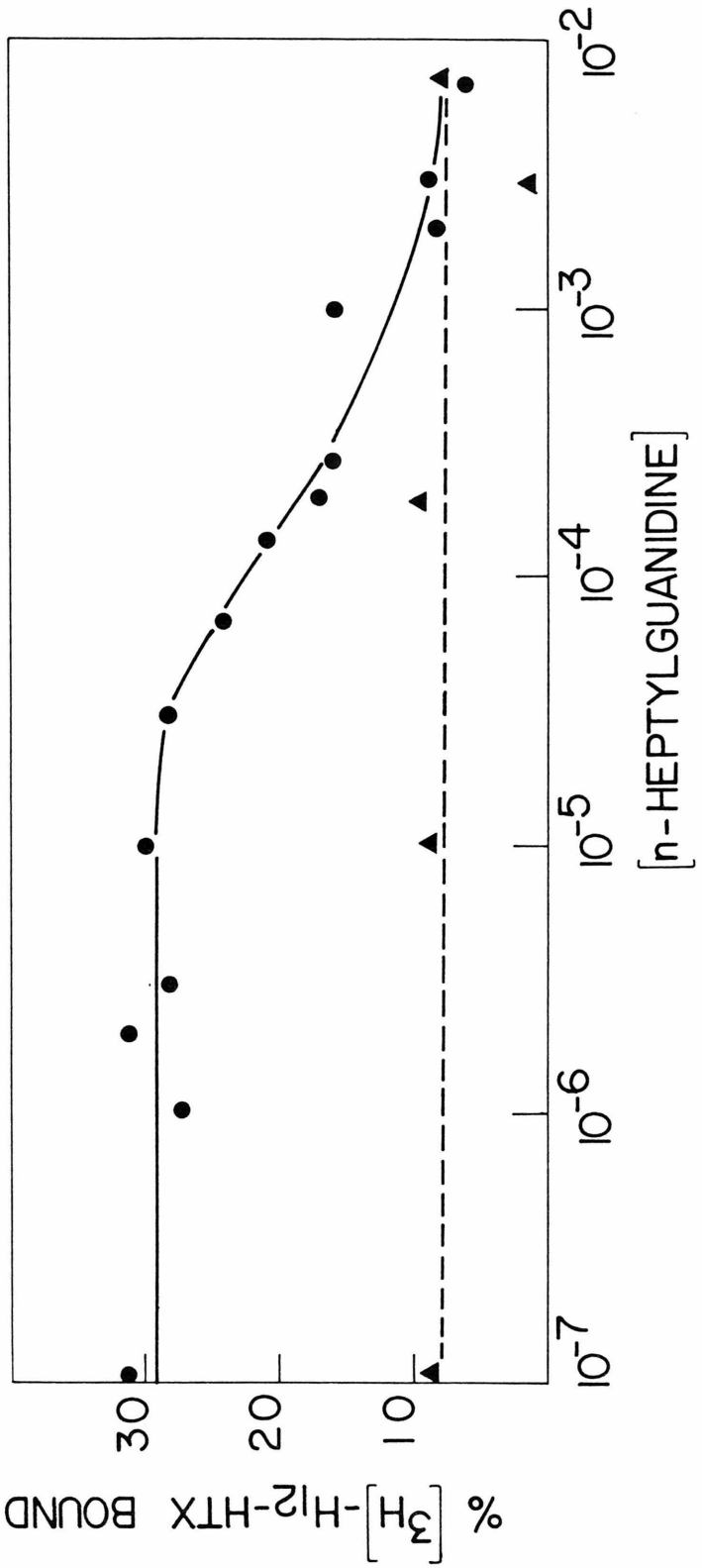

% [3H]-H12-HTX BOUND
30
20
10
10-7
10-6
10-5
10-4
10-3
10-2
[n-HEPTYLGUANIDINE]

Figure 5. A plot of log K_d versus chain length of linear alkylguanidines. The log of the K_d of the linear chain alkylguanidines was plotted against the number of carbon atoms in their sidechains. The two linear portions of the plot have slopes of -900 cal/carbon atom and -2400 cal/carbon atom. The K_ds of neopentylguanidine, cyclohexylguanidine and octylamine are shown on the line for illustration purposes; they were not used in calculating the placement on slope of the lines.

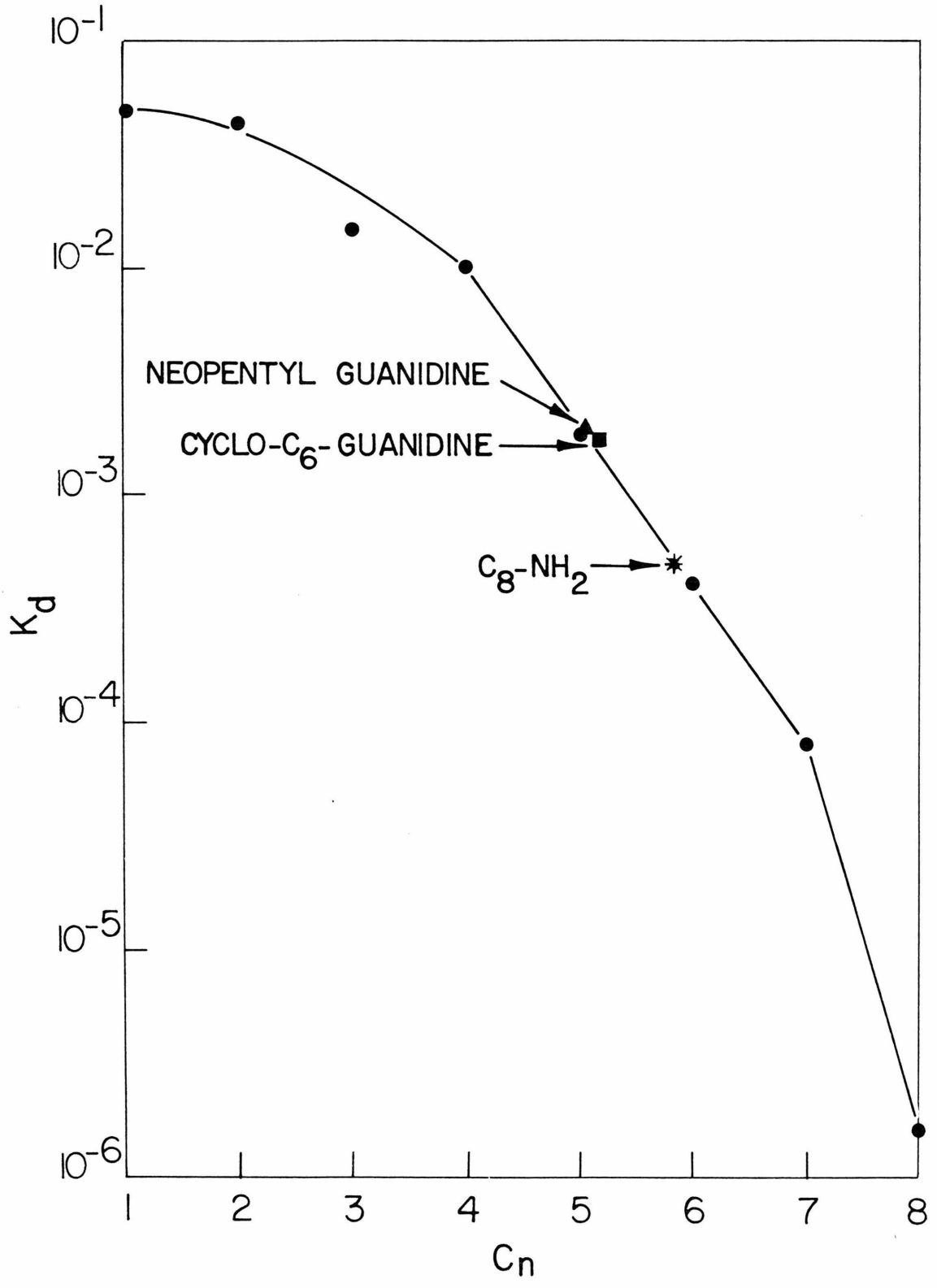

10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
K_d
NEOPENTYL GUANIDINE
CYCLO-C_6- GUANIDINE
C_8-NH_2
1
2
3
4
5
6
7
8
Cn

Figure 6. The competition of neopentylguanidine with [^{3}H]-H_{12}-HTX binding. AcChR enriched membrane fragments (1.5 μM in α-BuTx sites) were titrated with neopentylguanidine. The membrane fragments were suspended in Torpedo Ringer's buffer with 0.5 μM [^{3}H]-H_{12}-HTX (●-●-●) or 0.5 μM [^{3}H]-H_{12}-HTX plus 44 μM H_8-HTX (▲-▲-▲). After 30 minutes of incubation at room temperature, the membrane fragments were pelleted and the amount of radioactivity remaining in the supernatant was determined.

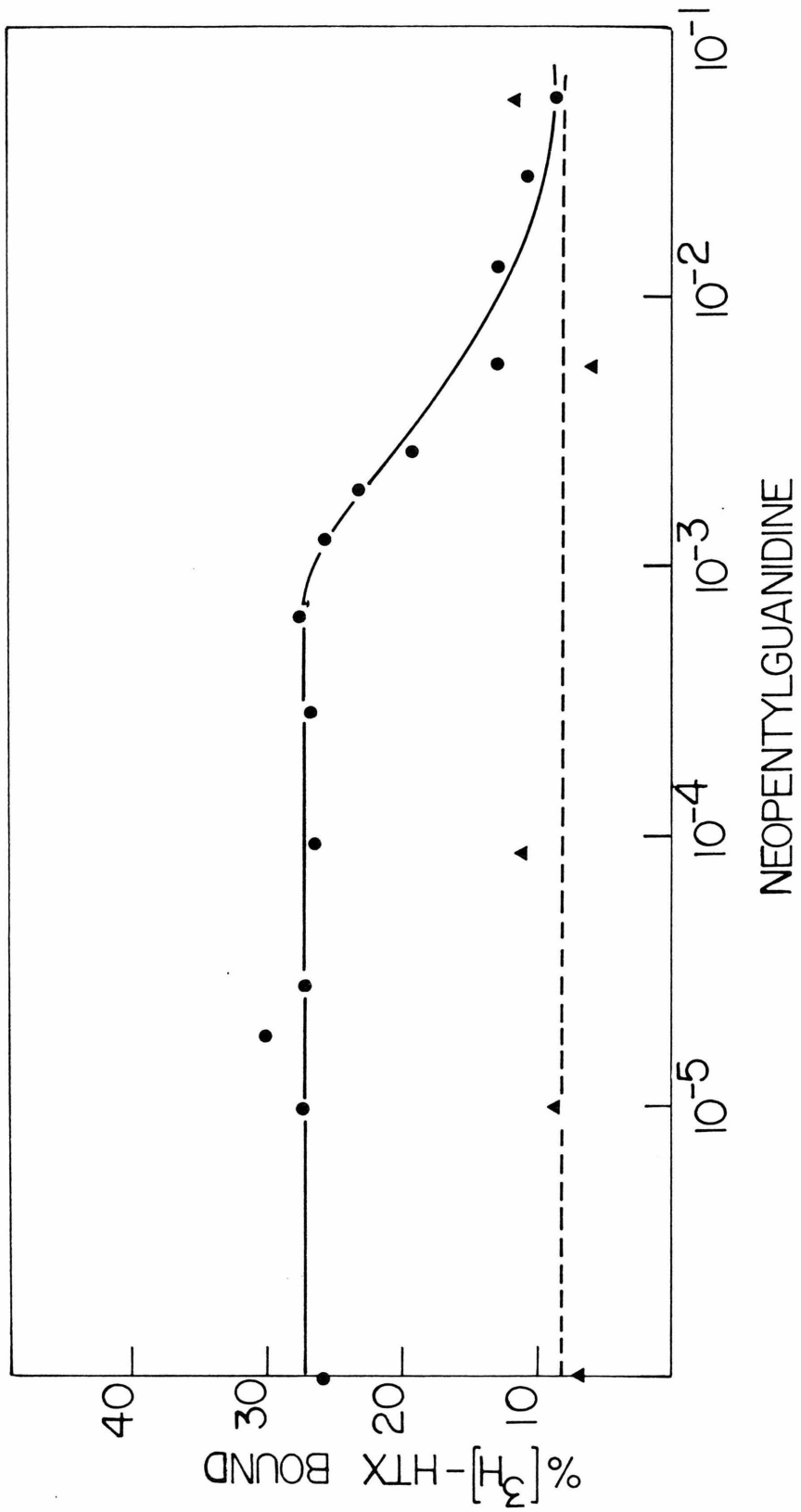
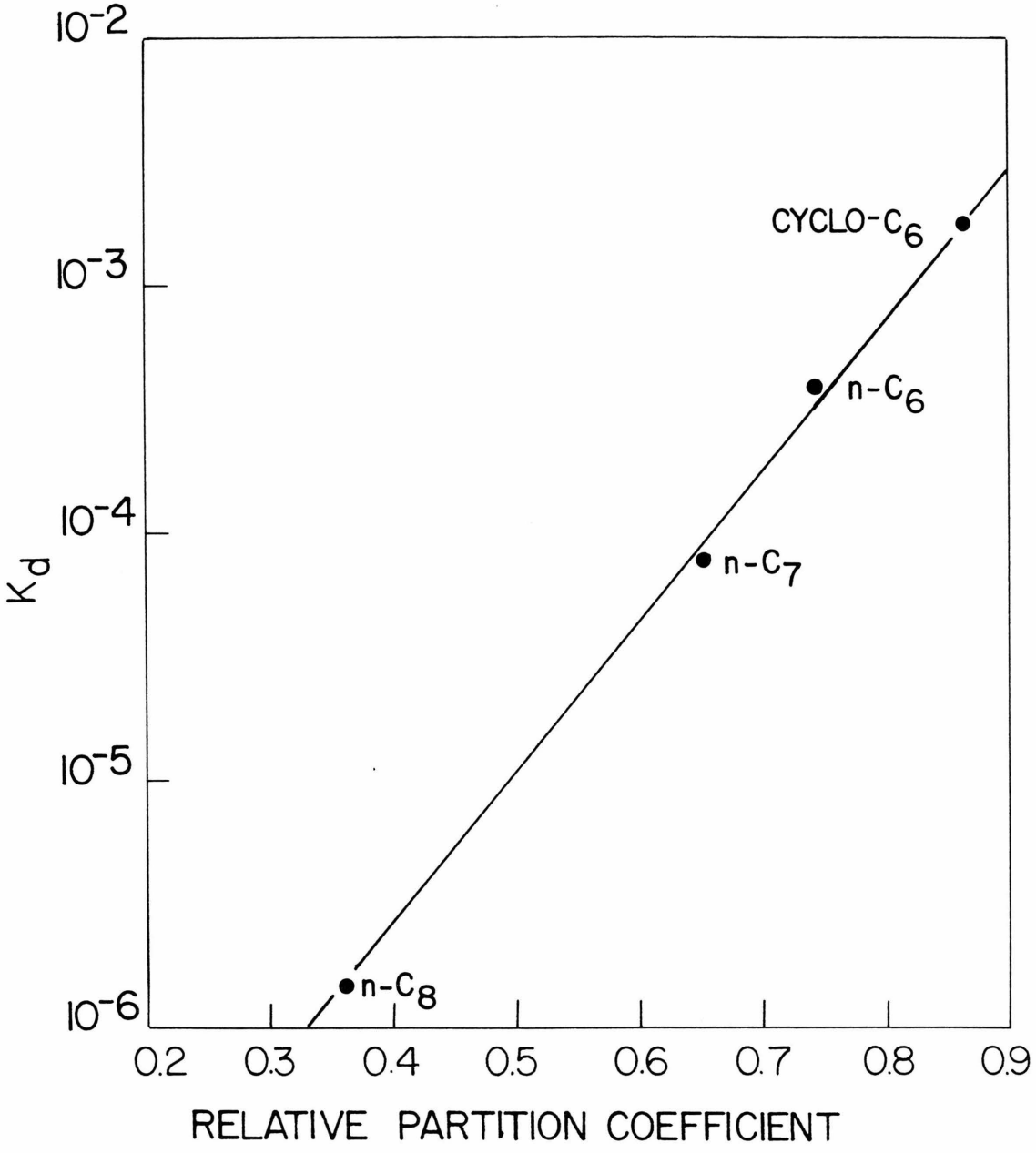

40
30
20
10
% [3H]-HTX BOUND
10-5
10-4
10-3
10-2
10-1
NEOPENTYLGUANIDINE

Figure 7. A plot of log K_d versus relative partition coefficient of some alkylguanidines. The membrane fragments (∿2 mM lipid, 2 μM α-BuTx sites) were incubated 30 minutes with the alkylguanidine (10^{-4} M) of interest, then pelleted. The concentration of alkylguanidine remaining in the supernatant was divided by the concentration before pelleting to give the relative partition coefficient. The value is not the true partition coefficient, as the exact concentration of lipid accessible to the alkylguanidine was not known.


10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
K_d
CYCLO-C_6
n-C_6
n-C_7
n-C_8
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
RELATIVE PARTITION COEFFICIENT

buffer and membrane fragments were determined and it was found that a plot of log K_d vs. relative partition coefficient was a straight line, even with inclusion of cyclohexylguanidine Fig. 7). The absolute partition coefficients could not be determined because of the uncertainty as to what concentration of membrane lipid was truly accessible to the amphipathic ligands. Studies with alkylguanidines were also undertaken in which these compounds were competed with [^{3}H]-Carb (Fig. 8). It was found that these ligands exhibited a moderate affinity for the AcChR agonist binding site. No chain length dependence was observed in the range tested (one through five carbon atoms) (Table II). A previous study using spin-labeled acylcholines (Bienvenue, et al., 1977) reported that there was a correlation between hydrophobicity and ligand affinity. However, the acylcholines used had very long (16 or 18 carbon atoms) side chains. It is possible that only the distal ends of these side chains were partitioning into the membranes.

The binding properties of the HTX derivatives examined in this study are informative as to the structure of the binding site. Perhydrohistrionicotoxin has a structure in which the number two position of azaspiro undecane system is substituted with a pentyl group (Fig. 1, I). Binding studies in which compounds II, III (Fig. 9) and both stereoisomers of V were competed with [^{3}H]-H_{12}-HTX demonstrated (Table III) that the stereochemistry of the 2-position substituent did not affect the affinity of the ligand. Omission of the pentyl group from

Figure 8. The competition of n-pentylguanidine with Carb binding. AcChR enriched membrane fragments (0.6 μM in α-BuTx sites) were titrated with n-Propylguanidine. The membrane fragments were suspended in Torpedo Ringer's with 0.14 μM [^{3}H]-Carb. After 30 minutes incubation at room temperature, the membrane fragments were pelleted and the amount of radioactivity remaining in the supernatant was determined.

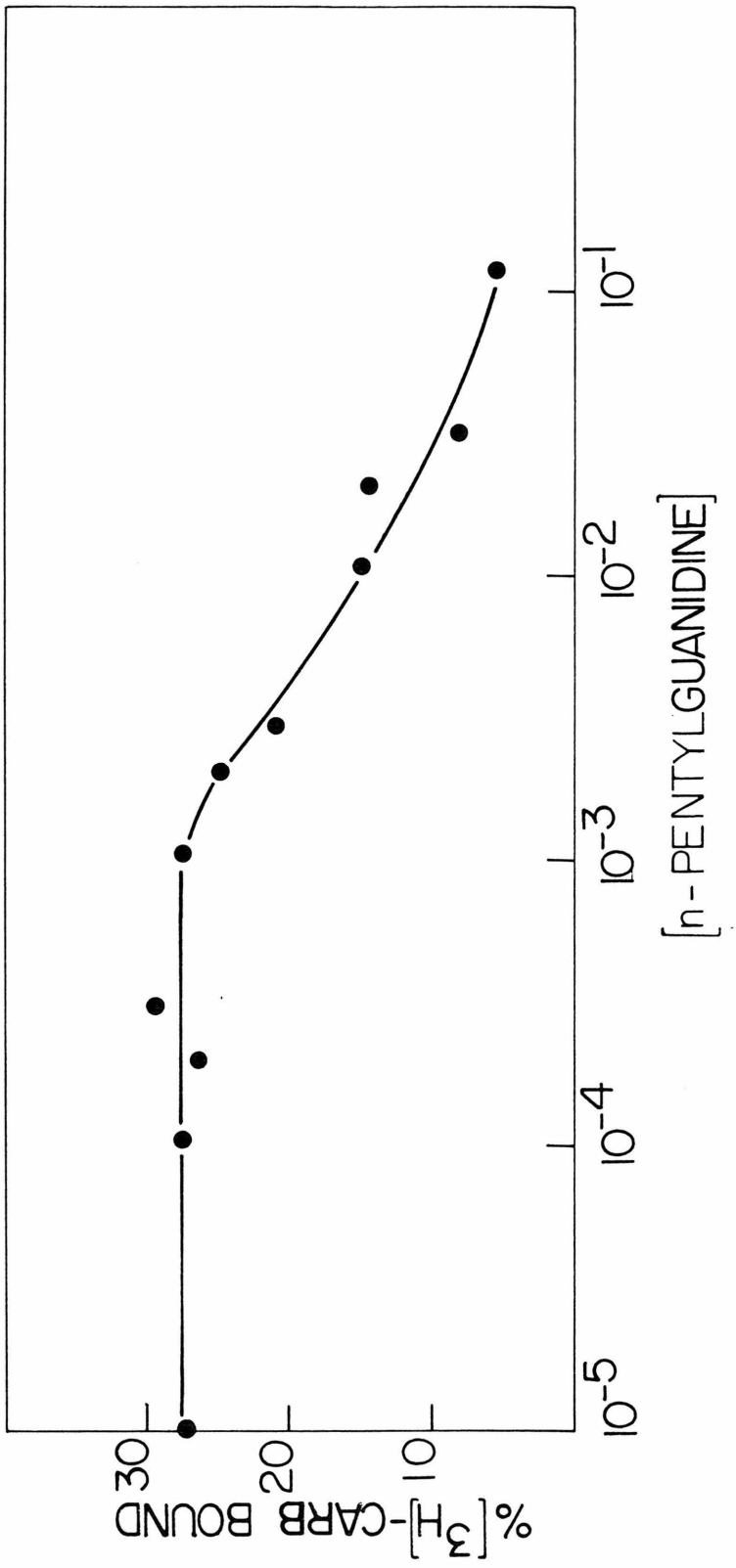
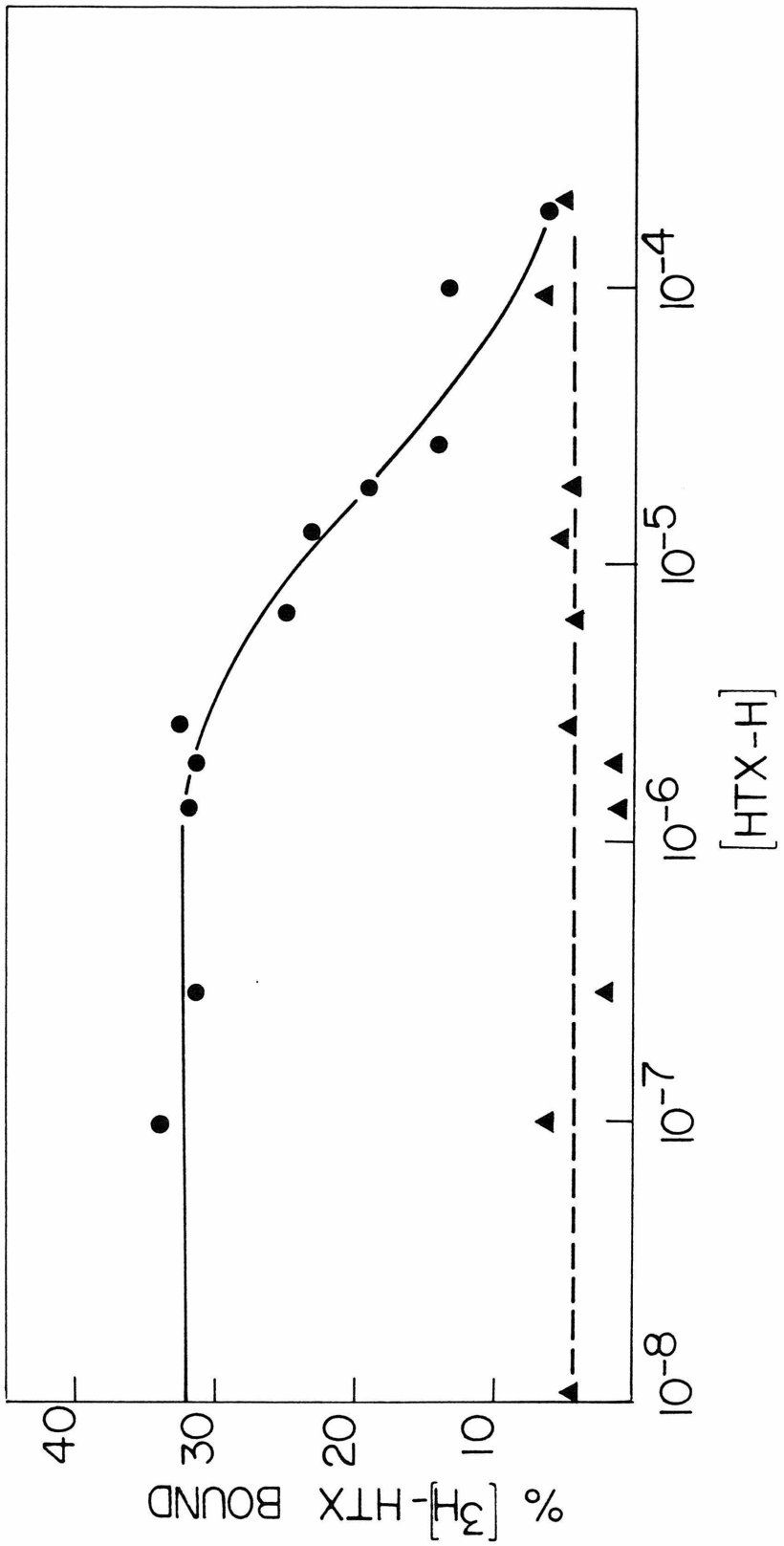

% [3H]-CARB BOUND
30
20
10
10-5
10-4
10-3
10-2
10-1
[n-PENTYLGUANIDINE]

Figure 9. The competition of HTX-H with [^{3}H]-H_{12}-HTX binding. AcChR enriched membrane fragments were titrated with HTX-H. The membranes (1.5 μM in α-BuTx sites) were suspended in Torpedo Ringer's with 0.5 μM [^{3}H]-H_{12}-HTX (●-●-●) or 0.5 μM [^{3}H]-H_{12}-HTX + 44 μM H_8-HTX (▲-▲-▲). After 30 minutes in incubation at room temperature, the membrane fragments were pelleted and the amount of radioactivity remaining in the supernatant was determined.


% [3H]-HTX BOUND
40
30
20
10
10-8
10-7
10-6
10-5
10-4
[HTX-H]

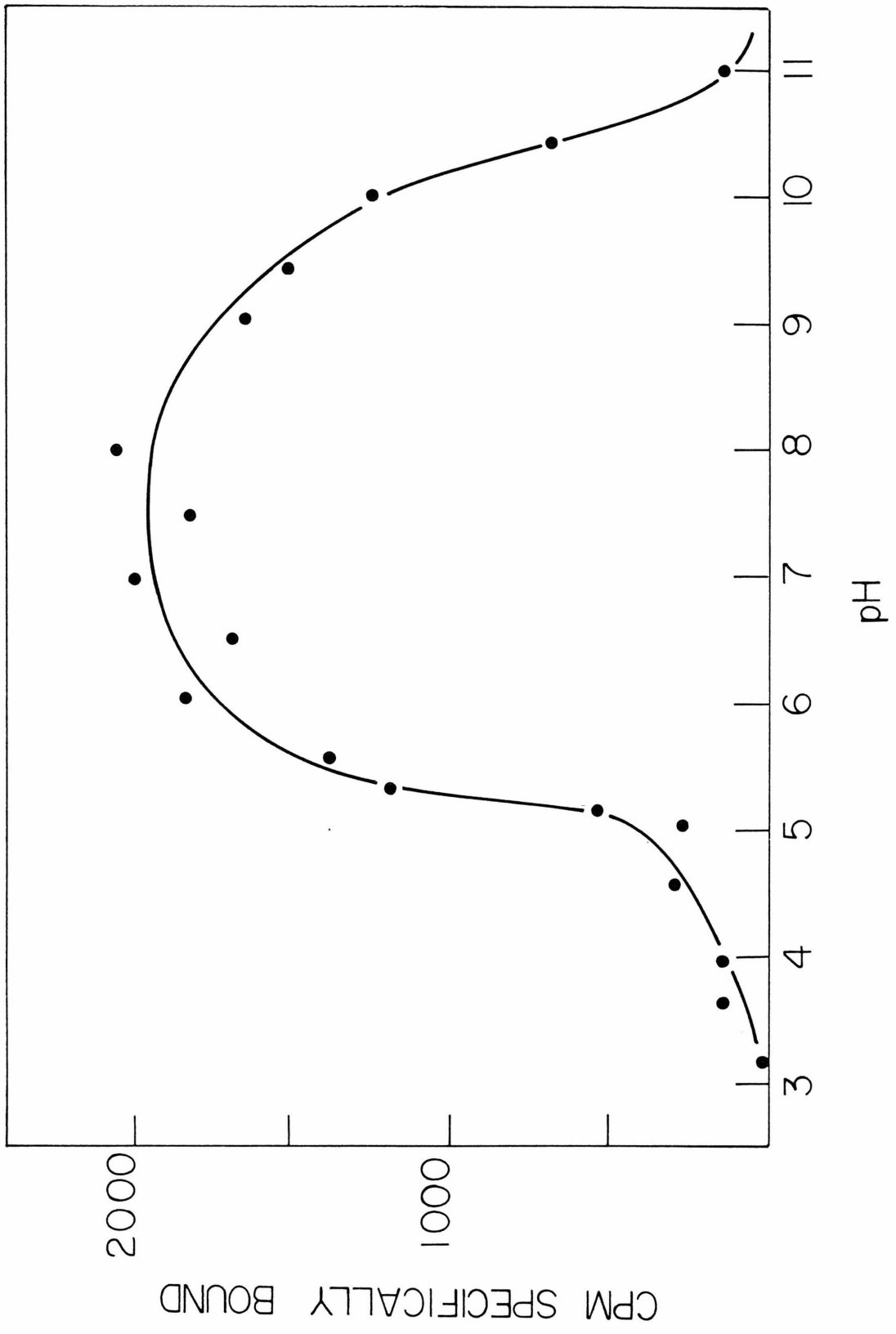
the toxin structure or modification of the group into a structure with a terminal hydroxyl group substantially affected the binding constant. The affinities of compounds II and III were more than thirty-fold lower than the affinity of H_{12}-HTX (Table II), corresponding to a loss of ΔG of 2.1 kcal/mole when calculated by using the formula $G = -RT \ln K_{eq}$.

Binding assays with compound V, dehydroxy-HTX, showed that its affinity was surprisingly high; being only 3-fold lower than H_{12}-HTX (Table III). This result was quite unexpected as this derivative is missing the hydroxyl group at the number 8 position which was presumed to be vital for HTX binding. It should be noted, however, that this derivative is much more hydrophobic than H_{12}-HTX and, as will be discussed, hydrophobicity appears to be a vital parameter in determining a ligand's affinity for the HTX site.

II. pH Dependence of HTX Binding

Figure 10 demonstrates the pH dependence of [^{3}H]-H_{12}-HTX binding (from Elliott, et al., 1980). It is apparent that the midpoint of the high pH decrease in binding is 10.1. The shape of this plot is consistent with the ionization of a single group and the value of pK_a determined is consistent with that of an amino group, probably that of HTX itself. The loss of binding activity at low pH is very rapid and is probably due to an effect on more than one group and/or to denaturation of the AcChR. It was hoped that a study correlating K_d vs. pH would allow determination of the pK_a of the AcChR group responsible for HTX

Figure 10. [^{3}H]-H_{12}-HTX binding versus pH. AcChR enriched membrane fragments (1.2 μM in α-BuTx binding sites) suspended in buffers of various pHs but constant Na^+ content were mixed with 0.3 μM [^{3}H]-H_{12}-HTX. After 20' incubation the membrane fragments were pelleted and the amount of radioactivity in the supernatant assayed. A parallel experiment was done in which 40 μM H_8-HTX was added to the samples to saturate all specific HTX binding. Specific [^{3}H]-H_{12}-HTX binding was then calculated and plotted against pH.


CPM SPECIFICALLY BOUND
2000
1000
3
4
5
6
7
8
9
10
11
pH

binding. A slight (2x) weakening of binding was observed as pH was decreased to 6.0, with the decrease beginning at 6.5. Unfortunately, below pH 6.0 a loss of binding sites was observed (Fig. 11) and the data vital to the pK_a determination were unobtainable. We can state only that the pK_a of the group is less than 6.5.

III. The Binding of Dansylguanidine (Fig. 1; VII)

Dansylguanidine was synthesized in the hope that it would serve as a fluorescent probe of the HTX site suitable for use in stopped-flow spectroscopy. As it is quite hydrophobic a high binding affinity was predicted and this was found to be the case; the K_d was 27 μM when assayed using competition with [^{3}H]-H_{12}-HTX.

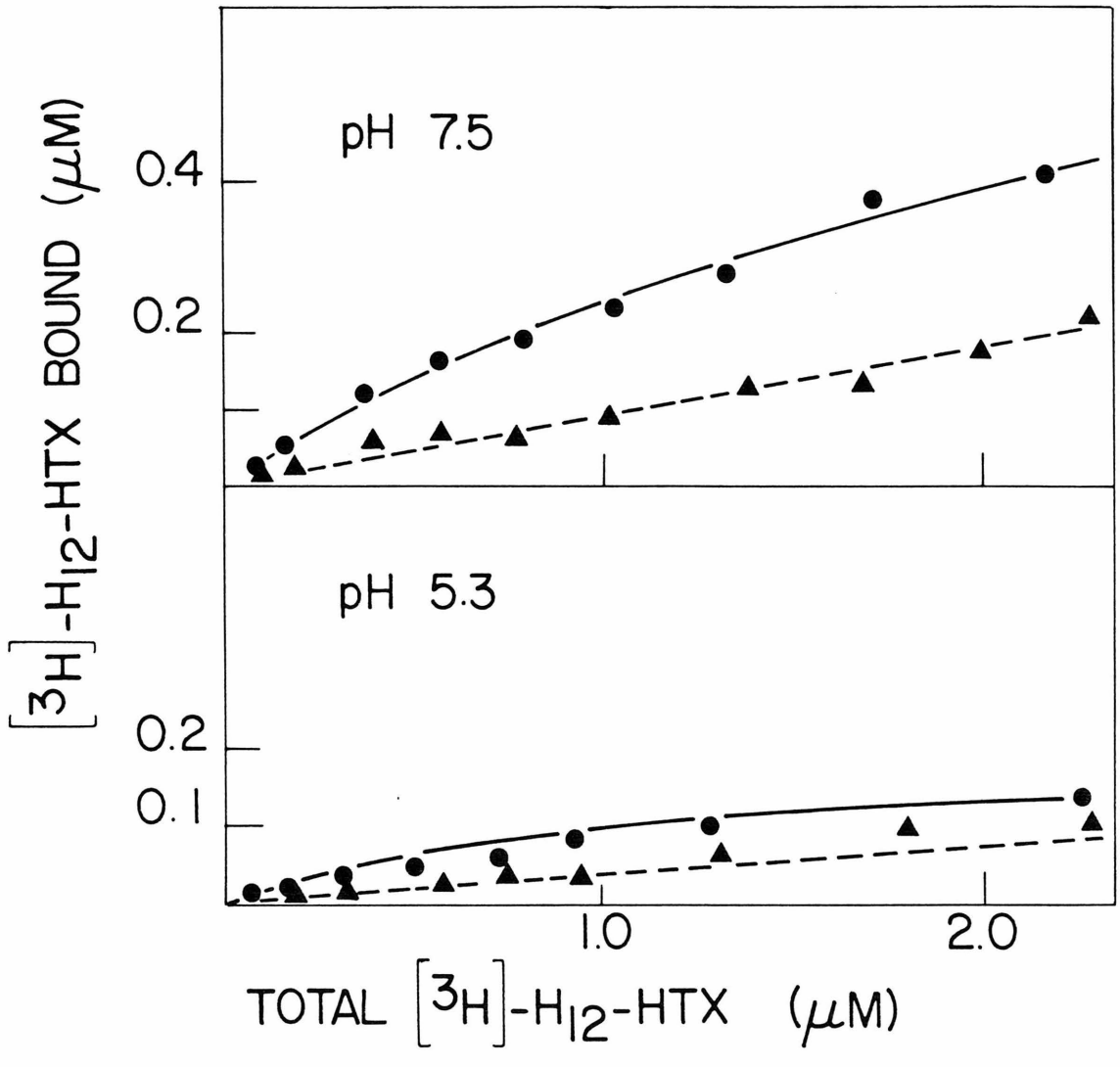
A very large (∿100 X) increase in fluorescence accompanied by a blue shift of ∿30 nm in the amines on moderation was found when the ligand was mixed with AcChR enriched membrane fragments. When H_8-HTX was added in large excess, the fluorescence intensity was decreased only slightly (∿10%).

Attempts at using the ligand as a probe in the stopped-flow spectrometer failed, however. At least four phases were apparent, with rates which ranged from 150 s^{-1} to 0.03 s^{-1}. These phases were not altered upon addition of octylguanidine and it was thought that all were related to partitioning of the ligand into the membrane. The signal from these phases

Figure 11. [^{3}H]-H_{12}-HTX binding titrations at pH 7.5 and pH 5.3.

a) AcChR enriched membrane fragments (1.4 μM in α-BuTx binding sites) were suspended in pH 7.5 buffer. [^{3}H]-H_{12}-HTX was added in increasing concentration to each successive aliquot and allowed to incubate 20' minutes at room temperature. The membrane fragments were then pelleted and the radioactivity remaining in the supernatant was determined. A parallel titration was done in in which 40 μM H_8-HTX was added to the aliquots to saturate all specific HTX binding.

b) The same experiment as in A, except that pH 5.3 buffer was used. The concentration of AcChR is identical to that in Section a.


pH 7.5
pH 5.3
0.4
0.2
0.2
0.1
1.0
2.0
[3H]-H12-HTX BOUND (μM)
TOTAL [3H]-H12-HTX (μM)

apparently obscured any signal due to specific binding of ligand to the AcChR site responsible for HTX binding.

IV. Labeling of the AcChR with [^{3}H]-H_{12}-HTX-N_3 (Fig. 1, VIII)

As is shown in Fig. 11, [^{3}H]-HTX-N_3 labeled three of the four protein subunits present in the alkali treated membranes. Proteins of molecular weight 40,000, 50,000, and 65,000 daltons were labeled in ratios of approximately 4:1:1 respectively, when labeled with 6 μM [^{3}H]-HTX-N_3. This concentration of HTX-N_3 is approximately twice the K_d (Figure 15). Higher proportions of the 40,000 dalton protein were labeled as the concentration of HTX-N_3 was increased. When H_8-HTX was added in large excess (Fig. 14), all labeling of the 65,000 dalton protein was blocked, while the labeling of the 40,000 and 50,000 dalton proteins was only partially blocked. The residual labeling in both these subunits decreased by proportionally the same amount. Preincubation with Carb did not alter the labeling significantly (Fig. 13). Preparations which contained the 90,000 dalton and 43,000 dalton subunits showed large amounts of label incorporation into the 90,000 dalton protein. Labeling experiments with this and other hydrophobic photoaffinity labels (Blanchard and Raftery, 1979) have been plagued with large amounts of what is presumably non-specific binding which makes careful control of the labeling conditions critical. It should be noted that in a typical experiment, 95% of the label initially mixed with the membrane fragments was removed in the two washing steps prior to application to the gel. This

was presumably unbound ligand and ligand which reacted with water after photolysis. Of the remaining 5%, 98% was found ahead of the dye front, which is the position in which phospholipids electrophorese on SDS gels. This left 0.1% of the total label used actually bound to protein.

Figure 12. Labeling of alkali treated AcChR enriched membrane fragments with HTX-N_3. AcChR enriched membrane fragments were treated with alkali such that their final specific activity was 3.2 mmoles of α-BuTx sites per mg of protein. These membranes were then mixed in the dark with 7.8×10^{-4} M [^{3}H]-HTX-N_3 and Torpedo Ringer's buffer to give final concentrations of 1.9 μM α-BuTx sites and 5.6 μM [^{3}H]-HTX-N_3. After 30 minutes of incubation in the dark, the mixture was transferred into a 2 ml cylindrical quartz curvette with a small stirring bar inside. This was then irradiated 15 minutes with a UVSL-25 ultraviolet light (Ultraviolet Products Co., San Gabriel, California). The membranes were pelleted and resuspended twice with changes of buffer each time.

Then membrane fragments corresponding to 0.6 mg of total protein were loaded onto a 1 x 10 cm SDS-polyacrylamide gel and electrophoresed. The gel was then sliced, digested and counted.

The positions of the 65, 60, 50, and 40 x 10^3 dalton subunits are shown based on Rf values determined with a gel of the same preparation stained with Coomassie Brilliant Blue.

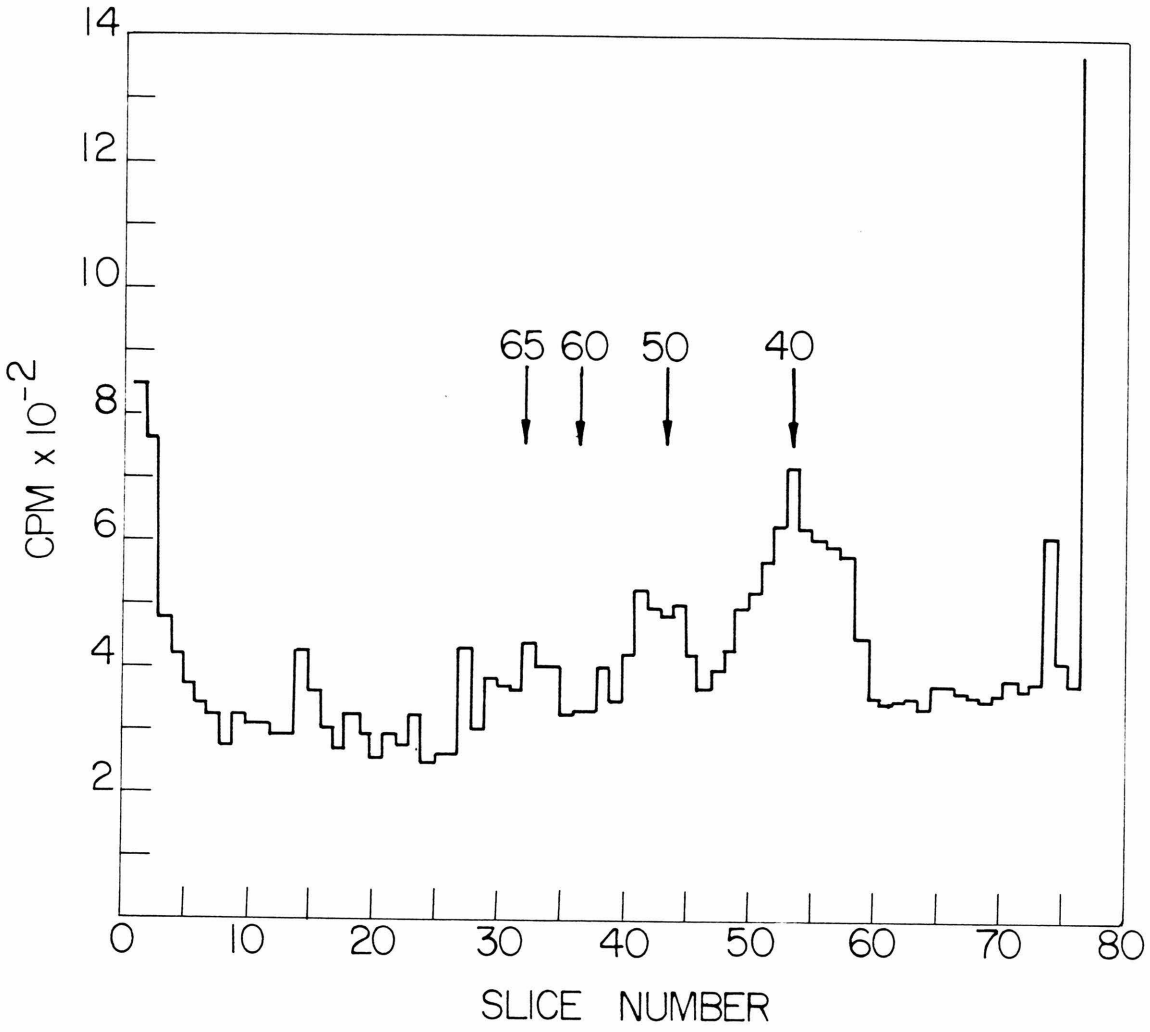

14
12
10
8
6
4
2
CPM x 10⁻²
65
60
50
40
0
10
20
30
40
50
60
70
80
SLICE NUMBER

Figure 13. The effect of Carb on the labeling of alkali treated AcChR enriched membrane fragments with HTX-N_3. The same protocol as described in the caption to Fig. 12 was used, except that the membrane fragments were incubated with 1 μM Carb 5 minutes prior to addition of [^{3}H]-HTX-N_3. The membrane fragment preparation used was the same as that used in the experiments described in Figs. 11 and 14.

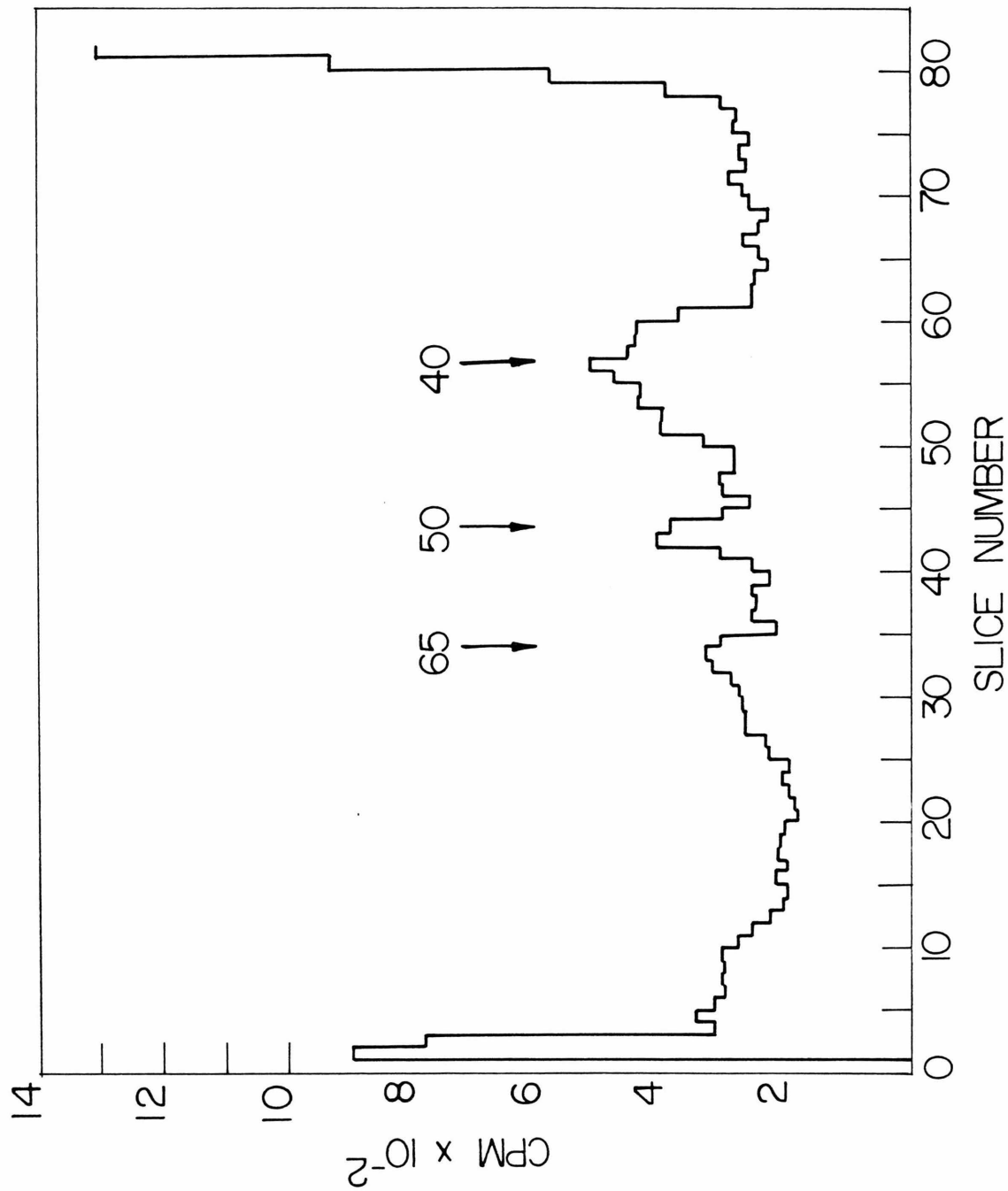

CPM x 10⁻²
14
12
10
8
6
4
2
0
10
20
30
40
50
60
70
80
SLICE NUMBER
65
50
40

Figure 14. Labeling of alkali treated membrane fragments with [^{3}H]-HTX-N_3 in the presence of H_8-HTX. The same protocol as described in the caption of Fig. 12 was followed, except that 100 μM H_8 was also included in the membrane fragment and [^{3}H]-HTX-N_3 mixture. The same membrane fragment preparation was used as in the experiments described in Figs. 11 and 13.

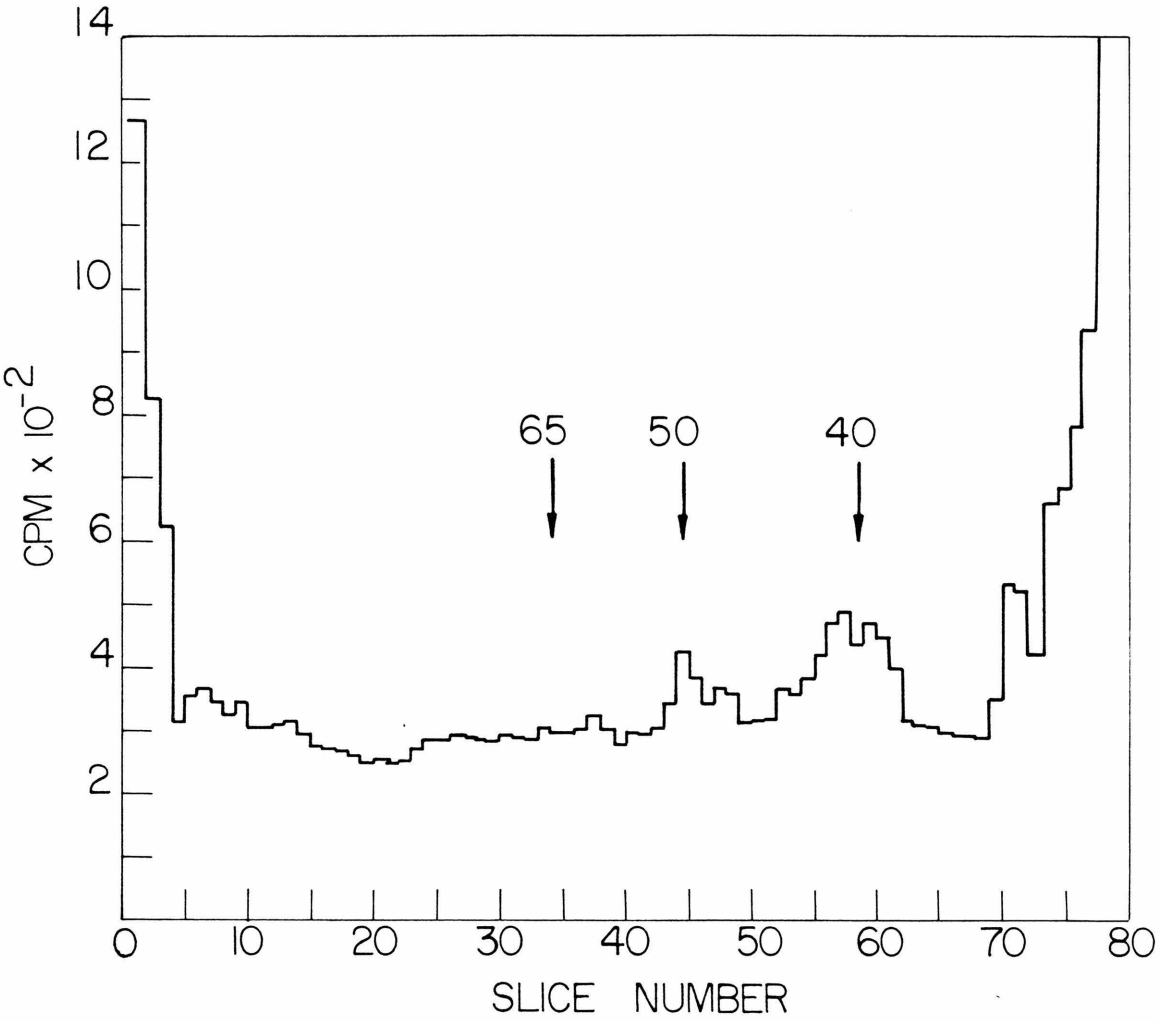
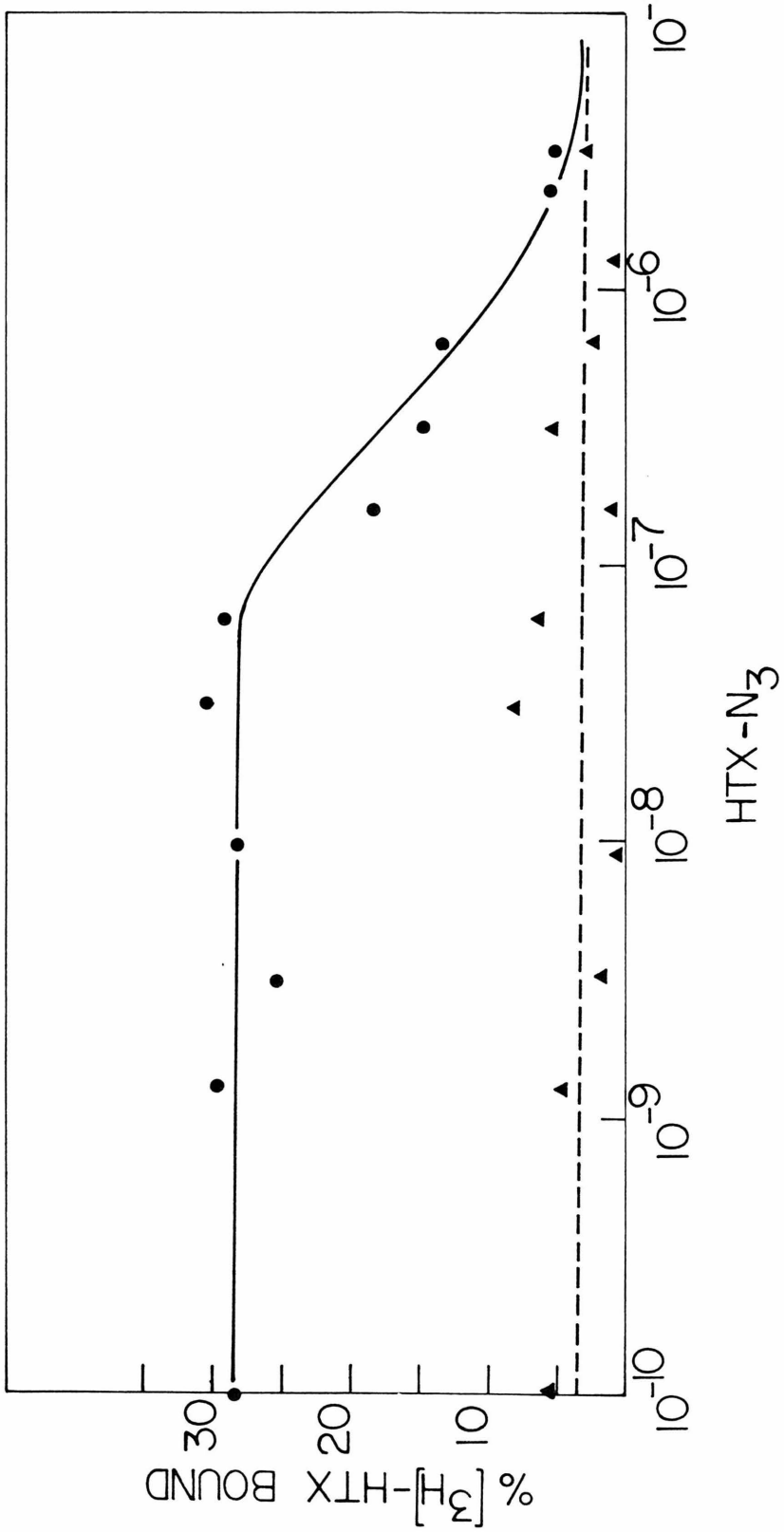

14
12
10
8
6
4
2
CPM x 10^{-2}
65
50
40
0
10
20
30
40
50
60
70
80
SLICE NUMBER

Figure 15. The competition of HTX-N_3 with [^{3}H]-H_{12}-HTX binding. AcChR enriched membrane fragments were titrated with HTX-N_3. The membrane fragments (1.4 μM in α-BuTx sites) were suspended in Torpedo Ringer's buffer with either 0.5 μM [^{3}H]-H_{12}-HTX (●-●-●) or 0.5 μM [^{3}H]-H_{12}-HTX plus 44 μM H_8-HTX (▲-▲-▲). After a 30 minute incubation, the membrane fragments were pelleted, and the amount of radioactivity remaining in the supernatant was determined.


% [3H]-HTX BOUND
30
20
10
10-10
10-9
10-8
10-7
10-6
10-5
HTX-N3

DISCUSSION

The above results (Fig. 10) have indicated that HTX must be ionized in order to bind to the AcChR. Further evidence of this is that octylamine competes with [^{3}H]-H_{12}-HTX for the AcChR site, whereas octanol, with a headgroup capable only of forming a hydrogen bond, competes weakly, if at all, even though long chain alcohols are known to act as anesthetics (Seeman, 1975). Octylguanidine, possessing both a charged group and a possible donor group for hydrogen bonding, binds much more tightly than octylamine, as would be expected if the AcChR site had two functional groups responsible for HTX binding. The difference in affinities corresponds to a difference in binding energies of 3.5 kcal/mole, which is consistent with the loss of a hydrogen bond.

Among the several possible conformations for HTX, the one in which the azaspiro ring is in the chair conformation uniquely allows superposition of the charged HTX amino group and the hydroxyl group, which is capable of forming a hydrogen bond to a suitable acceptor group, with two analogous groups of guanidine (i.e., the two nitrogens of a resonance form). A possible counter group for binding such a moiety would be a carboxylate ion, which meets steric requirements and possesses suitable charged and H-bond forming atoms. The chair conformation also results in an HTX molecule which is hydrophobic on one side and hydrophilic on the other. The hydrophobic portion of the alkylguanidine molecule can be superimposed with portions of the

hydrophobic regions of HTX and still allow superposition of the hydrophilic groups.

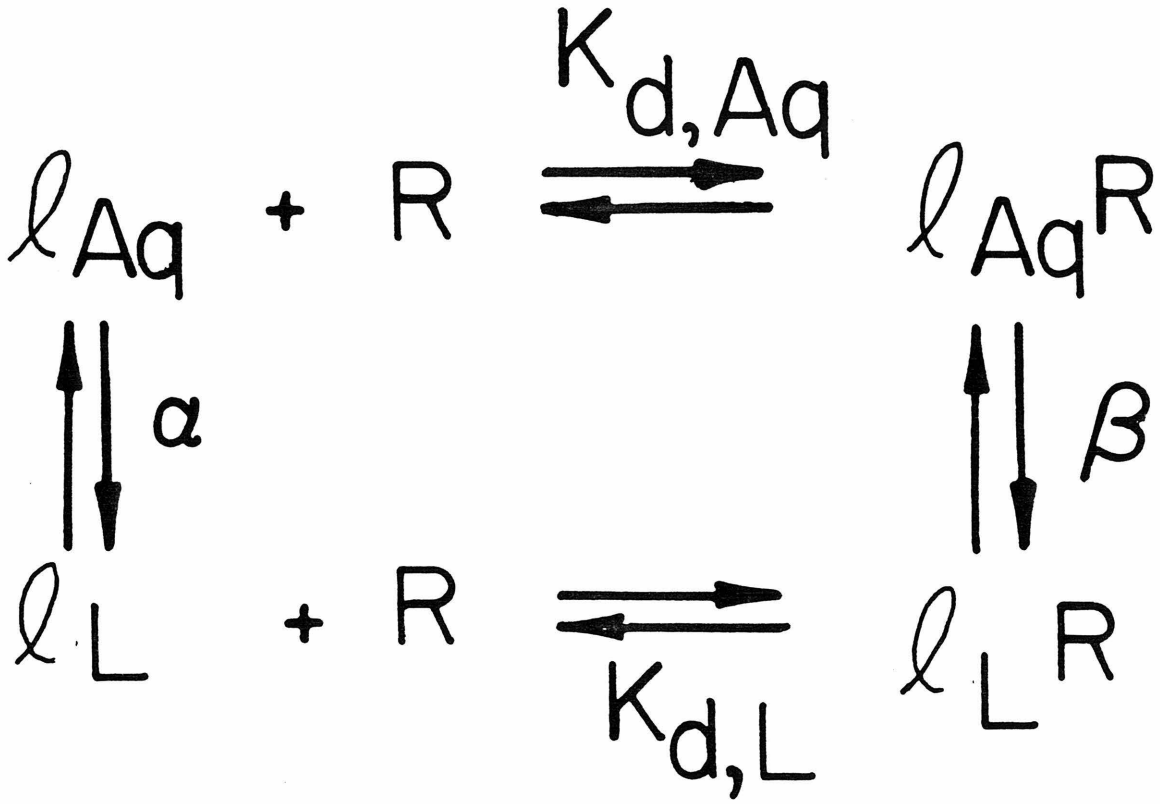
The more hydrophobic alkylguanidines inhibited HTX binding better than those with short alkyl chains (Fig. 5). The plot of log K_d vs. chain length has characteristics expected of a plot of partition coefficient vs. chain length. Short chain alkylguanidines, in which the alkyl chain was within the hydration sphere of the headgroup, showed only a slight chain length dependence. Increasing the length gave linear dependence of log K_d vs. length until heptylguanidine. The slope of this linear region is about -900 calories per carbon atom, in excellent agreement with the -884 calories per carbon atom expected for transfer of an aliphatic chain from water to a hydrocarbon (McAuliffe, 1966; Tanford, 1973). The change in slope beyond this point is not typical of a hydrocarbon:water partition coefficient dependence. This may be due to a property of the membrane, which only approximates a hydrocarbon solvent. It is unlikely to be due to micelle formation because of the low concentrations involved (Reed and Trzos, 1979) and also the constant amount of non-specific [^{3}H]-H_{12}-HTX binding throughout the titration, which suggests that the integrity of the membranes has not been disrupted.

The discontinuity in Fig. 5 at the higher alkylchain lengths is removed if the partition coefficents measured experimentally are used in place of simple chain lengths (Fig. 7). This dependence of K_d upon what is a membrane:ligand interaction

implies that the membrane plays a key role in the binding of alkylguanidines to the AcChR. This could be explained if the hydrophobic ligands partitioned into the membrane prior to binding (Fig. 16). This partitioning would serve to create an enriched phase from which the ligand would bind to the receptor. Derivatives such as methylguanidine would probably bind only from the aqueous phase and hence be capable of less binding interaction. This model proposes that the membrane itself is part of the HTX binding site, serving to form a fluid hydrophobic region capable of accommodating variously shaped hydrophobic groups. It is possible that the membrane:alkylguanidine partition coefficients may fortuitously mimic the hydrophobic interaction between the ligand and a hydrophobic site of the protein, though it is difficult to imagine a protein hydrophobic site which could provide strong, predictable interactions for linear alkyl chains, cyclic chains and HTX with its large hydrophobic surface on the side opposite its binding groups. This is especially true in light of the lack of dependence on the stereochemistry of the number two position of the HTX azaspiroundecane system.

The complete loss of the pentylgroup attached to the number 2 carbon results in a loss in binding energy (ΔG) of 2.0 kcal/mole as calculated by the formula $\Delta G = RT \ln K_{eq}$. This is in only qualitative agreement with the above model, as the energy of transfer for a pentyl group from water to hydrocarbon may be calculated (Tanford, 1973) to be -5.7 kcal assuming only one terminal methyl group. The loss of 2.2 kcal of binding energy

Figure 16. A possible model for ligand binding to the HTX site. The ligand partition coefficient between the aqueous and membrane lipid phases is denoted by α, while β represents partitioning of the AcChR bound ligand between aqueous and lipid phases. β is presumably a function of α.


$K_{d,Aq}$
ℓ_{Aq} + R
$\ell_{Aq}R$
α
β
ℓ_L + R
$\ell_L R$
$K_{d,L}$

incurred by adding a terminal hydroxyl group to the pentyl chain is also qualitatively consistent with the model, as the ΔG of transfer of pentyl alcohol from pure alcohol to water has been determined (Kinoshita et al.,1958) to be 3.2 kcal, a value which should be higher for transfer from hydrocarbon to water. The disparity between the observed values for ΔG and those calculated from assumptions based on transfer of pure aliphatic compunds into pure hydrocarbon phases may be due to incomplete transfer of the groups in question or to perturbations of the membrane structure induced by the bulk of the HTX molecule.

Based on the relative K_ds of octylamine and octylguanidine, I expected a large loss of binding energy for dehydroxyl-HTX (V) as compared to H_{12}-HTX. This turned out not to be the case (Table III) and, in light of the model, I feel this is best explained by the increased hydrophobicity of the dehydroxy-HTX, which was noticeably less water soluble than H_{12}-HTX.

The competition between Carb and alkyl guanidines was weak, with K_ds almost 10^4 higher than the K_d of Carb for the agonist site. I believe the binding is due to the simple charge-charge interaction seen at the agonist site with other positively charged molecules (Elliott, et al., 1980. It has been reported (Narahashi, 1980) that methyl guandidine inhibits from end plate potentials with a K_I of about 1 mM and this is in good agreement with the value of K_d seen for Carb inhibition with this molecule. Octylguanidine was reported to have a K_I of about 1 μM in the same study, a value which is in agreement with

the 1.5 μM K_d I have determined for octylguanidine by competition with [^{3}H]-H_{12}-HTX.

I attempted to determine the pK_a of the AcChR group responsible for HTX binding. Due to denaturation of the AcChR below about ph 6.0, I can report here only that the pK_a is below 6.5. Thus the data do not allow estimation of the hydrophobicity of the environment of the group in question.

The results with alkylguanidines are similar to results previously reported for interaction of alkylguanidines with the Na^+ channel of E. electricus (Reed and Trzos, 1979), in which the same dependence of K_d upon chain length was observed. A similar chain length dependence was observed when alkyl quaternary ammonium ions were used to inhibit conductance in K^+ channels (Armstrong, 1971). It is interesting that in the cases of 3 different ion transport channels involved in neurotransmission, all possessed a sensitivity to a positively charged amphipathic molecule that was affected by the chain length of the hydrophobic portion of the ligand. I found no competition between TTX and [^{3}H]-H_{12}-HTX binding to AcChR and no competition was found between H_8-HTX and [^{3}H]-TTX for the binding site on the voltage sensitive Na^+ channel (W. Agnew, personal communication). Thus direct homology between the sites on Na^+ channel and AcChR is not likely.

There is little to be said for the results of the dansylguanidine experiments. Any specific binding of the ligand was apparently obscured by the much larger

non-specific fluorescence enhancement which we attributed to partitioning of the dansylguanidine into the membrane. Since this compound should also interact with the TTX site of the sodium channel (Reed and Trzos, 1979), it would be interesting to see if the fluorescent properties of the ligand in these preparations make it a viable probe for stopped-flow spectroscopy.

[^{3}H]-HTX-N_3 labeled three of the four AcChR subunits (Fig. 12). However, H_8-HTX totally blocked binding only to the 65,000 dalton subunit (Fig. 14). Binding to the other two subunits labeled (the 40,000 and 50,000 dalton subunits) was also decreased to some extent by H_8-HTX, but this was probably due to competition of H_8-HTX and [^{3}H]-HTX-N_3 for a limited number of hydrophobic non-specific "sites". When 90,000 dalton protein was present in the preparations, it too was strongly labeled and this labeling was decreased by H_8-HTX. The 90,000 dalton protein was also labeled in experiments using procaineamide azide (Blanchard and Raftery, 1978), so it seems likely that this protein was labeled due to the presence of hydrophobic areas which non-specifically bind hydrophobic ligands. Removal of the 90,000 dalton protein has no known effect on AcChR function (see Chapter III). The amount of labeling to the 65,000 dalton and 50,000 dalton subunits was saturable, whereas incorporation of label into the 90,000 and 40,000 dalton subunits seemed proportional to the concentration of label up to the highest [^{3}H]-HTX-N_3 concentration used (28 μM). At 6 μM [^{3}H]-HTX-N_3, the optimal concentration, the 40,000 dalton band had four times

more label than either the 65,000 or 50,000 dalton bands which, given the 2:1:1 subunit stoichiometry of the AcChR, indicates that the 40,000 dalton subunit was binding twice as much label as either of the other two subunits labeled. The 60,000 dalton subunit was not labeled even when 28 μM [^{3}H]-HTX-N_3 was used.

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CHAPTER II

Proton magnetic resonance studies of cholinergic ligand binding to the acetylcholine receptor in its membrane environment.

INTRODUCTION

In recent years the application of NMR methodology to biological systems has yielded a wealth of information regarding structural and functional aspects of enzymes and proteins in solution. This has been achieved mainly by approaches such as direct observation of nuclei of constituent amino acid sidechains of the macromolecules or observation of perturbations of ligand nuclei exchanging between free and bound states under different sets of conditions. In order to extend such methods to the study of membrane-bound macromolecules, the second approach is the more feasible provided that (i) a high concentration of membrane-bound ligand binding sites can be obtained, (ii) a ligand is available that is readily observable by using current NMR instrumentation, and (iii) the ligand binding parameters are suitable for study of the exchange between free and bound states.

The third condition is not fulfilled by agonists such as acetylcholine (AcCh) and carbamylcholine (Carb), because these ligands have too high an affinity for the AcCh receptor (AcChR) under equilibrium conditions. However, all the necessary conditions are met by using choline as a ligand that binds to membranes highly enriched in the postsynaptic nicotinic AcChR. Titration of membrane fragments enriched in AcChR (Duguid and Raftery, 1973; Reed, et al., 1975; Elliott, et al., 1979) with choline, a known partial agonist (Adams and Sakmann, 1978), has allowed determination of the choline dissociation constant from the concentration dependence of the observed linewidth, because

nonspecific broadening was determined to be negligible by a similar titration of membrane fragments in which the AcChR agonist binding sites were blocked with α-bungarotoxin (α-BuTx). The temperature dependence of the dissociation constant and that of the methyl linewidth at a fixed choline concentration contained the data necessary for determination of the enthalpy of binding and for estimation of the apparent dissociation rate constant and the respective activation energy. Thus, it was possible to apply a highly informative spectroscopic technique to the study of membrane-bound receptors for elucidation of structural and kinetic properties.

MATERIALS AND METHODS

Membrane fragments enriched in AcChR were prepared using sucrose density centrifugation with a Beckman VTi-50 (Elliott, et al., 1979) rotor, as described in Chapter I. The enriched membranes were then centrifuged at 100,000 x g, and the pellet was resuspended in 0.01 M sodium phosphate buffer made with 99.7% ^2H_2O. After a minimum of 2 hours the membranes were again pelleted and resuspended in 0.01 M sodium phosphate buffer made with 100% ^2H_2O (Bio-Rad, Inc., Richmond, California). This last procedure was repeated and the preparation was then homogenized twice for 30 sec with a VirTis 23 homogenizer operating at maximal speed. The two hour equilibration periods were necessary to achieve the low levels of protiated water required in the experiments, as the membrane fragments retain considerable amounts of water in the bilayer. The final concentration of AcChR, expressed as [^{125}I]-α-BuTx binding sites was typically 10 μM.

The concentration of α-BuTx binding sites was determined using the disc assay (Schmidt and Raftery, 1973) in which complexes of AcChR and [^{125}I]-α-BuTx are adsorbed to DEAE-paper discs, which are then washed to remove uncomplexed α-BuTx and counted in a Beckman γ-Ray counter. Protein concentrations were determined using the method of Lowry (Lowry, et al., 1951).

The inhibition constant of a ligand for the agonist site can be determined by measuring the dependence of the initial rate of [^{125}I]-α-BuTx:AcChR complex formation (Blanchard, et al., 1979). At a time defined as zero, membrane fragments with a

given concentration of the agonist are mixed with [^{125}I]-α-BuTx and aliquots are taken at given time intervals. These aliquots are immediately applied to the DEAE paper disc, which quenches the complexing reaction. The discs are washed to remove uncomplexed [^{125}I]-α-BuTx and their content of radioactivity is determined. Thus a hyberbolic curve of radioactive α-BuTx bound versus time can be plotted and the initial rate determined in the presence of different agonist concentrations, this dependence can then be interpolated back to an inhibition constant for the agonist. This method was used to confirm the K_d for choline determined by NMR.

In order to minimize the content of protiated water in the stock solutions, the sodium chloride, choline and Carb used in the experiments were dissolved in 99.7% ^2H_2O and then lyophilized. Stock solutions were then prepared from these reagents using 100% $^2H_2$ and kept tightly sealed. At all stages of the titrations, care was taken to minimize atmospheric contact with the solutions in order to maintain low levels of protiated water. These low levels were necessary as the nuclear magnetic resonance signals being observed were quite close to the resonance frequency of the proton in $^2HO^1H$ and were hence easily obscured if the protiated water content was too high. Excessive protiated water content also caused dynamic range problems with the spectrometer.

^1H NMR spectra were recorded using a Varian XL-100-V15 spectrometer in the Fourier transform mode locked on the ^2H_2O

signal of the sample. The temperatures were controlled using a Varian variable temperature unit. Sample volumes between 1.5 to 2.0 ml were placed in 12 mm sample tubes. These small volumes necessitated the use of Teflon anti-vortex plugs (Wilmad, Inc., Buena, New Jersey).

RESULTS

Choline Titration

When a relatively low concentration of choline (200 μM) was added to a suspension of AcChR enriched membrane fragments at moderate concentration (10 μM in α-BuTx binding sites), the linewidth of the three equivalent choline methyl groups was observed to be substantially broader than that of choline alone in aqueous solution (Fig. 1, upper). At a ratio of choline to AcChR α-BuTx binding sites of 10:1, a wide (≈15 Hz) line was observed and, as the ratio of choline to AcChR was increased, the observed linewidth decreased until an apparently constant value was obtained when the choline to AcChR site ratio was on the order of 100:1. When the same experiment was performed with membrane fragments that had been treated with α-BuTx, a constant and narrow linewidth was observed, independent of the choline concentration. These observations are consistent with the hypothesis that the linebroadening was due to a specific interaction of choline with the AcChR. No change in chemical shift was observed, and it was considered that this quantity was the same for both bound and free states. In the case of weak binding (see below), the free species is the one observed and, if there is only one bound state, the equation describing the linewidth is:

$$\Gamma_{obs} - \Gamma_F = \frac{1_b/1_o}{\pi(T_{2b} + t_b)} \quad , \qquad (1)$$

where Γ_{obs} is the width of the observed lorentzian line at half

its height, Γ_{obs} is the width of the line under conditions in which the specific broadening is abolished, l_b/l_o is the fraction of the ligand bound (with free ligand in great excess over bound), and T_{2b} and t_b are the spin-spin relaxation time and average residence time of the bound ligand, respectively (Swift and Connick, 1962). At a given temperature, T_{2b} and t_b are constant, and the observed broadening is solely a reflection of the fraction of the ligand bound. Thus, the dissociation constant for the ligand may be obtained by plotting $(\Gamma_{obs} - \Gamma_F)^{-1}$ versus total ligand concentration, l_o, (Fig. 1, lower), because expressing l_b/l_o in terms of l_o and K_d results in:

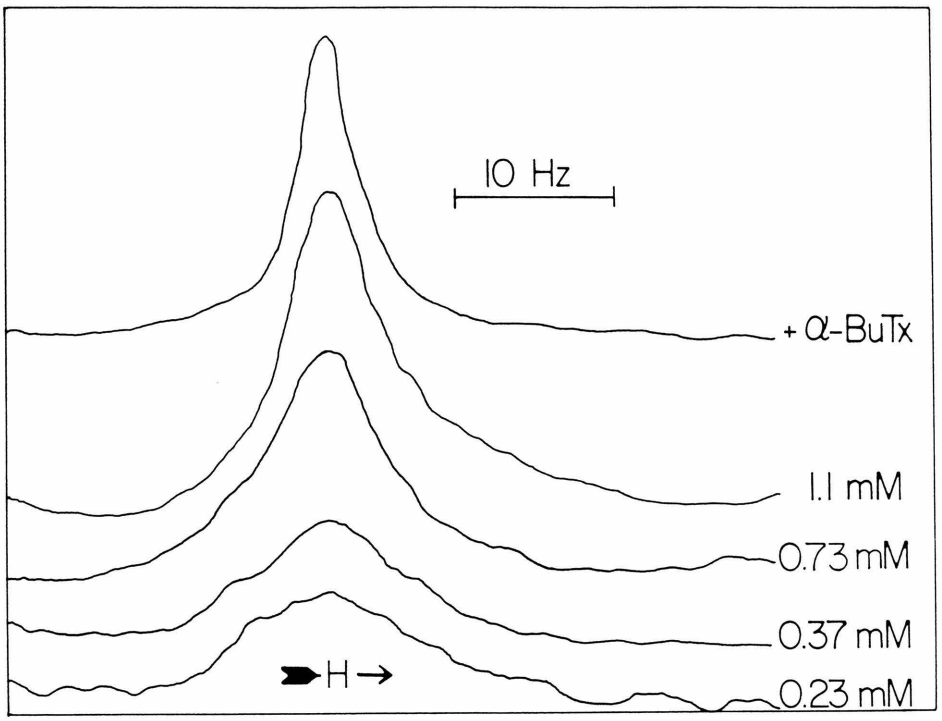
$$\frac{1}{\Gamma_{obs} - \Gamma_F} = \frac{\pi(T_{2b} + t_b)}{r_o}(l_o + K_d) \quad . \qquad (2)$$

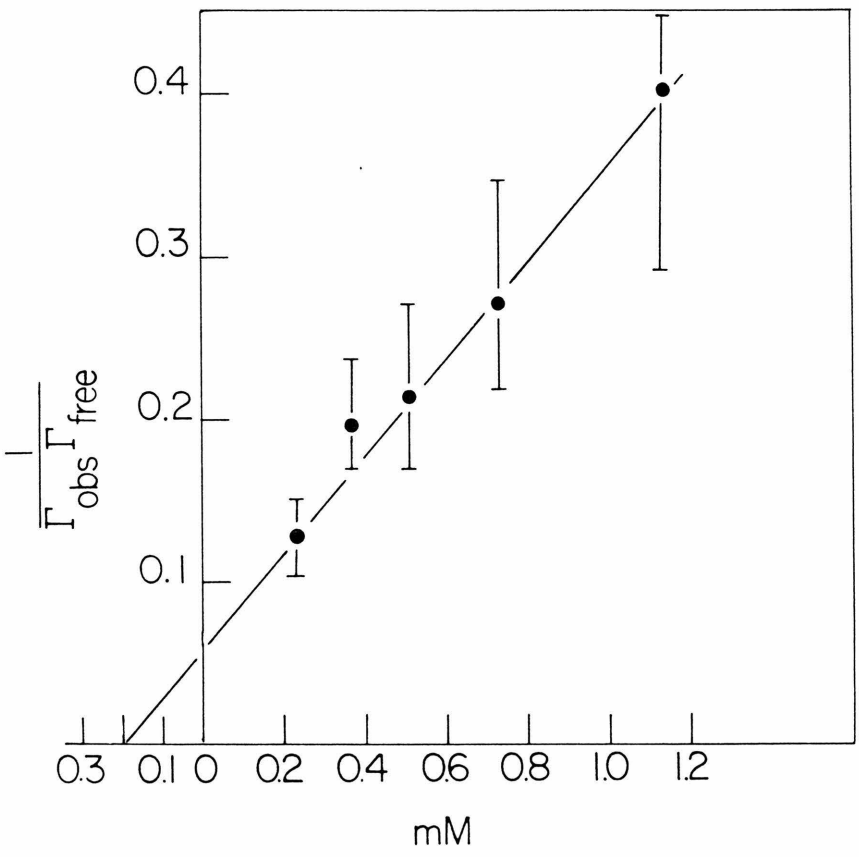
Here, r_o is the total concentration of binding sites and K_d is the apparent dissociation constant of the complex.

Several titrations of choline-receptor association at different temperatures were conducted to determine the thermodynamic parameters of the interaction between choline and the AcChR. For the equilibrium constant little or no temperature dependence was found within our limits of error (Table 1), and it was concluded that ΔH for this binding was small.

The temperature dependence of the slopes (S) of the semi-reciprocol plots, as in Fig. 1 lower, also contains important information:

Figure 1. (Upper) Proton magnetic resonance spectra of choline methyl groups at various concentrations of choline. All spectra were recorded in 10 mM P_i buffer (^2H_2O), pH 7.4, containing membrane fragments with 5 μM AcChR at 4.6°C. The top spectrum was recorded 20 min after the addition of 10 μM α-BuTx to the above solution in which the choline concentration was 1.1 mM. (Lower) Plot of the spectra in Upper by using Eq. 2; $(\Gamma_{obs} - \Gamma_F)^{-1}$ versus [choline]. The line was determined by a weighted linear least-squares fit.


10 Hz
+ α-BuTx
1.1 mM
0.73 mM
0.37 mM
0.23 mM
H


0.4
0.3
0.2
0.1
$\frac{1}{\Gamma_{obs} - \Gamma_{free}}$
0.3
0.1
0
0.2
0.4
0.6
0.8
1.0
1.2
mM

$$S = \frac{\pi}{r_o}(T_{2b} + t_b) \quad . \qquad (3)$$

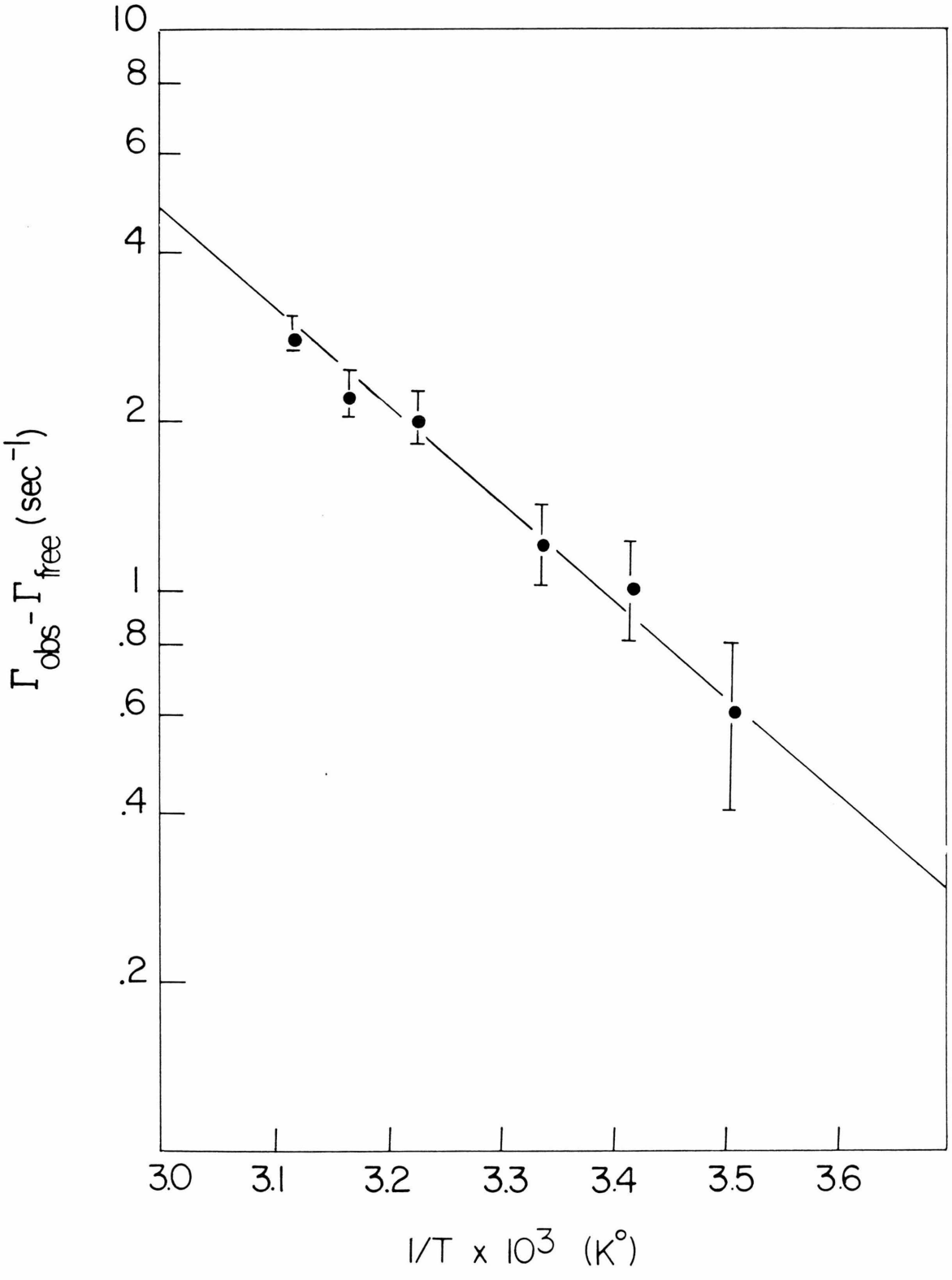
The two parameters, T_{2b} and t_b, are expected to have opposite temperature dependences (Sykes, et al., 1970; Dwek, 1973). We observed a decrease in slope with increasing temperature (see Table 1) and an increase in the observed linewidth at low saturation of the AcChR with choline. This indicates (see Discussion) that $t_b > T_{2b}$. Because t_b, the lifetime of the complex, is equal to $1/k_{diss}$, where k_{diss} is the (apparent) dissociation rate constant of the complex, one estimates from the data in Table 1 that $k_{diss} \geq 1.6 \times 10^3 S^{-1}$ at 18°C.

The temperature dependence of the slope (Eq. 3) yields the activation energy for k_{diss} with $E_a(k_{diss}) = 5 \pm 2$ kcal/mol. A statistically independent estimate of $E_a(k_{diss})$ is obtained from an Arrhenius plot of log $(\Gamma_{obs} - \Gamma_F)$ at a fixed choline concentration versus 1/T (see Fig. 2). Because K_d is essentially temperature independent in the temperature range studied (see above and Table 1), the observed slope in Fig. 2 reflects the activation energy of the process characterized by t_b, which in this case was found to be 5.5 kcal/mol. Both values for E_a are in good agreement.

Competition Studies

Competition studies with radiolabeled ligands have shown that different agonists apparently bind to the same site of the AcChR from Torpedo (Raftery, 1975; Eldefrawi and Eldefrawi, 1977) or Electrophorus (Fu, et al., 1977) membranes. Therefore,

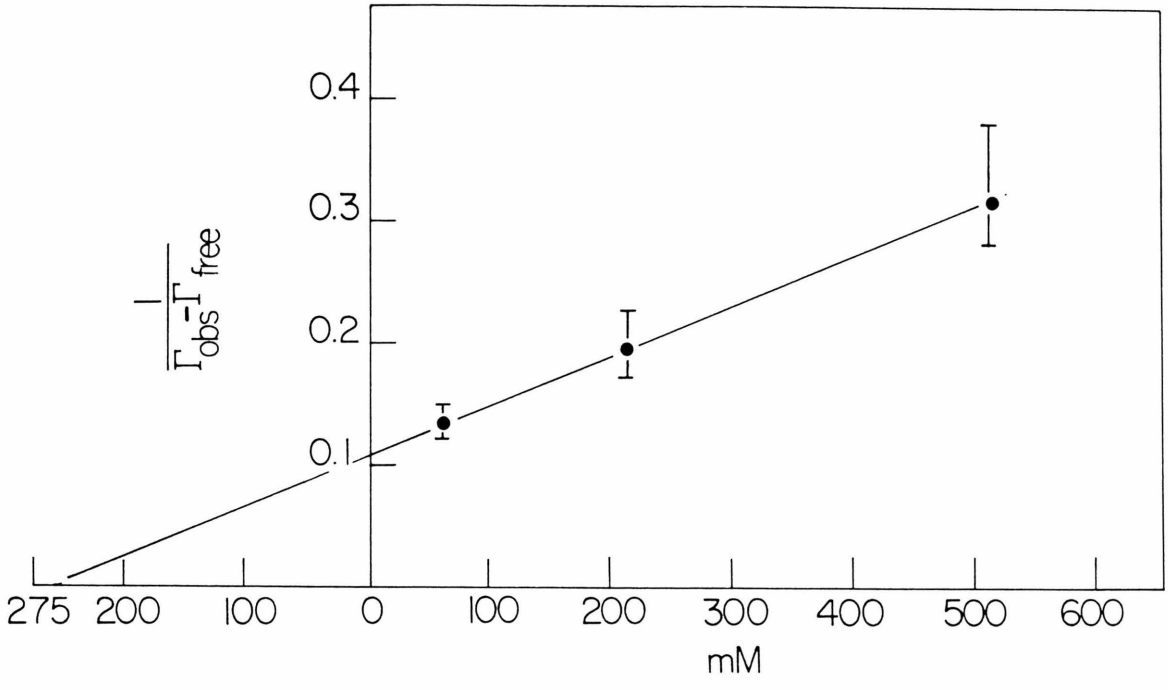
Figure 2. Arrhenius plot constructed by using data from proton magnetic resonance spectra of a mixture of 0.7 mM choline and AcChR enriched membrane fragments (^{125}I-labeled α-BuTx site concentration = 22 μM) recorded at various temperatures. The plot is of log ($\Gamma_{obs} - T_F$) versus 1/T and yields E_a = 5.5 kcal/mol.

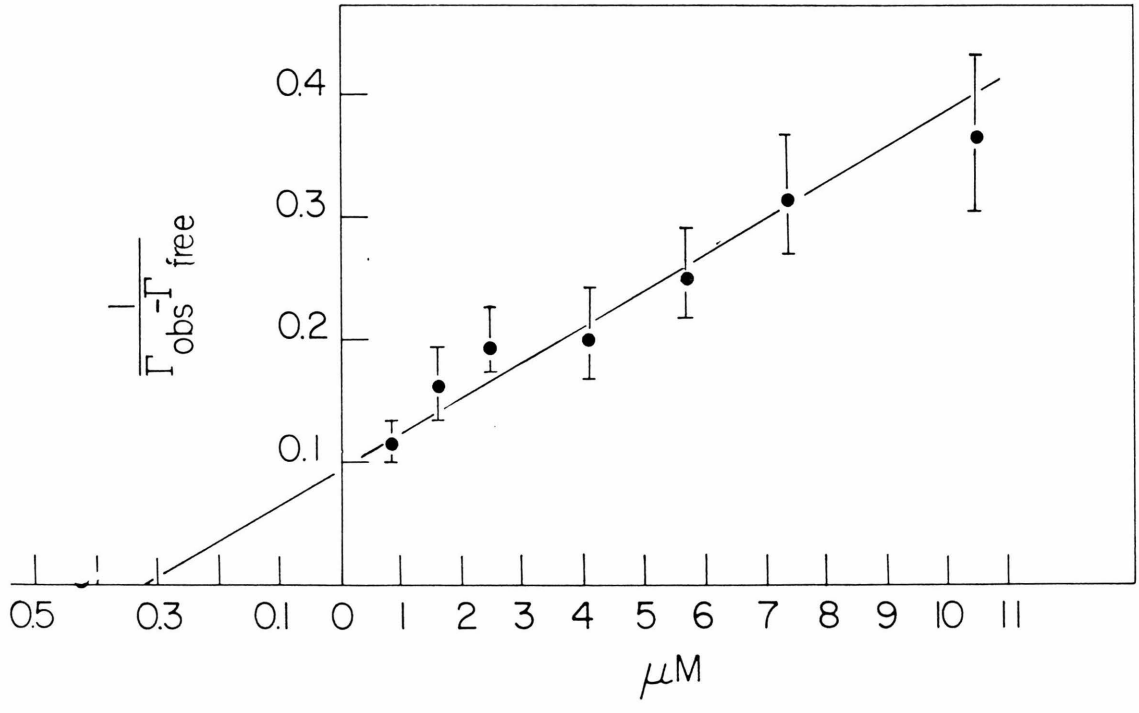

10
8
6
4
2
1
.8
.6
.4
.2
$\Gamma_{obs} - \Gamma_{free}$ (sec^{-1})
3.0
3.1
3.2
3.3
3.4
3.5
3.6
1/T x 10^3 (K°)

addition of the agonist Carb should reduce line broadening due to its interaction with the binding site of the ligand being observed (choline). Because Carb affinity for the AcChR is much higher than that of choline (Eldefrawi and Eldefrawi, 1977; Blanchard, et al., 1979; Schimerlik, et al., 1979; Quast, et al., 1979), a low concentration of Carb would be undetected in the NMR spectrum but would be expected to narrow the choline signal substantially. This behavior was observed, and a complete titration with Carb at fixed choline concentration (Fig. 3) resulted in an apparent K_d for Carb of 3.5 $\pm$ 0.1 μM when the data were plotted by using Eq. 2. This value must be corrected for the effect of choline competition by using the formula $K_d = K_{app}[1 + S_o/K_o]^{-1}$, where K_d is the true dissociation constant, K_{app} is the apparent dissociation constant measured, S_o is the concentration of the competing ligand (choline in this case), and K_o is the dissociation constant of this competing ligand. The corrected K_d for Carb was 0.5 $\pm$ 0.1 μM.

It has been shown that monovalent cations decrease the second-order rate constant for [^{125}I]-α-BuTx binding to (Schmidt and Raftery, 1974) and the affinity of the fluorescent antagonist 1,10-bis(3-aminopyridinium)-decane diiodide (Martinez-Carrion and Raftery, 1973) for detergent solubilized purified AcChR. Both these studies showed that monovalent cations have apparent binding constants of 5 mM. On the other hand sodium chloride, in the 5-50 mM range, had little or no effect on the binding of the agonist AcCh to solubilized

Figure 3. (Lower) Titration of choline-AcChR complex with Carb. The experiment monitors the variation of the choline linewidth with Carb concentration. The mixture contained AcChR-enriched membrane fragments (20 μM ^{125}I-labeled α-BuTx sites) and 0.9 mM choline in 10 mM P_i buffer (^2H_2O), pH 7.4, at 15°C. The plot shows $(\Gamma_{obs} - \Gamma_F)^{-1}$ versus [Carb]. The line is a weighted linear least-squares fit, giving an apparent K_d of 3.5 ± 0.1 μM. (Upper) Titration of choline-AcChR complex with NaCl. The conditions are as in Lower, with $(\Gamma_{obs} - \Gamma_F)^{-1}$ versus [NaCl] plotted. The line is a weighted least-squares fit, giving an apparent K_d of 260 ± 10 mM.


0.4
0.3
0.2
0.1
$\frac{1}{\Gamma_{obs}-\Gamma_{free}}$
275 200 100 0 100 200 300 400 500 600
mM


0.4
0.3
0.2
0.1
$\frac{1}{\Gamma_{obs}-\Gamma_{free}}$
0.5 0.3 0.1 0 1 2 3 4 5 6 7 8 9 10 11
μM

purified AcChR (T. Moody and M.A. Raftery, unpublished results). By using the same methods employed for Carb, we obtained a K_i (apparent) for NaCl of 260 $\pm$ 10 mM, indicating a much weaker effect of salt on the binding of the partial agonist choline than was observed on the binding of antagonist.

The dissociation constant for choline was also determined by inhibition of the kinetics of ^{125}I-labeled α-BuTx-AchChR association (Blanchard, et al., 1979) in buffer of composition identical to that used in the NMR experiments. The value obtained, 120 $\pm$ 50 μM, was in good agreement with that obtained from the NMR data (190 $\pm$ 65 μM, Table I). The same experiment performed in buffer made in protiated water resulted in essentially the same K_d.

DISCUSSION

The studies described are based on observation of a perturbation in the ^{1}H-NMR spectrum of a partial agonist induced by binding interactions with the AcChR in its native membrane environment. The effect is specific because it can be completely blocked by pretreatment of the membranes with α-BuTx, which specifically competitively blocks ligand binding to the AcChR (Quast, et al., 1978). The binding of α-BuTx should not affect any linebroadening due to non-specific interactions, and the data for Γ_F therefore include all linebroadening except that due to specific ligand binding at the AcChR agonist binding site.

The data were analyzed according to Eq. 1, which is a special case of a more general formula derived by Swift and Connick (Swift and Connick, 1962) from modified Bloch equations containing terms for chemical exchange (McConnell and Berger, 1957). The main assumptions leading to Eq. 1 are (i) there are only two kinds of environment for the nucleus (i.e., bound and free), (ii) the free nuclei are always in excess over the bound species and are therefore the ones observed, and (iii) there is no change in chemical shift due to binding.

Assumptions ii and iii were experimentally fulfilled in that we did not observe a chemical shift difference between bound and free ligand, and the concentrations of ligand binding sites used were $R_o \approx 10\ \mu M \ll K_d \approx 200\ \mu M$, so that at all ligand concentrations only a small fraction of the total ligand present was bound. Assumption i was made for the sake of mathematical simplicity because the original formulae of Swift and Connick

are complex and would not allow unambiguous determination of the many parameters they contain.

Plotting the observed half-width of the resonances according to Eq. 2 directly yielded the equilibrium constant, K_d. The value of 190 $\pm$ 65 μM for choline is in agreement with that obtained from inhibition of the kinetics of ^{125}I-labeled α-BuTx binding in the same medium (120 $\pm$ 50 μM). These values are more than 2 times higher than those determined in 4 mM protiated buffer containing calcium, where a value of 50 $\pm$ 4 μM was found using inhibition of [^{125}I]-α-BuTx binding (Blanchard, et al., 1979) and a value of 25 μM was found using the fluorescent probe ethidium (Schimerlik, et al., 1979; Quast, et al., 1979). A similar effect was observed with Carb, for which a K_d of 0.5 $\pm$ 0.1 μM was observed from competition with choline in the NMR experiments reported here, using calcium-free buffer. This value compares well with the value of 0.33 $\pm$ 0.05 μM determined by using radioactively labeled Carb in calcium-free buffer (Schimerlik and Raftery, unpublished). However, values for K_d of 0.05 - 0.12 μM were reported when the determinations were done in calcium containing buffers using several experimental procedures (Raftery, et al., 1975; Quast, et al., 1978; Quast, et al., 1979, Schimerlik, et al., 1979).

The finding of similar equilibrium constants for choline by inhibition of [^{125}I]-α- BuTx binding kinetics in protiated and deuterated buffers seems to indicate that the lack of calcium is responsible for the high K_d values observed. This

conclusion is in agreement with the observation (Cohen, et al., 1974) that calcium increases the affinity of the membrane bound AcChR for cholinergic ligands. Choline titrations at different temperatures showed essentially no temperature dependence of the equilibrium constant, and the enthalpy of binding was estimated to be < 1.5 kcal/mol. Therefore, binding of choline to AcChR is mainly entropy driven [$\Delta S \simeq 20 J/(mol \cdot K)$]. The increased entropy of the complex (compared to the free components) could be due to higher internal flexibility in the complex or a decreased exposure of the hydrophobic surfaces to the solvent. A ligand-induced conformational change of the AcChR provides a plausible explanation for both cases. There is independent evidence for such an agonist induced conformational change of the AcChR (Grünhagen, et al., 1977; Quast, et al., 1978; Schimerlik, et al., 1979), though this is the first report of a change in entropy accompanying agonist binding to membrane-bound AcChR. With solubilized purified AcChR from Electrophorus, a considerable positive change in enthalpy ($\Delta H \simeq$ + 20-30 kcal/mol) was observed which was overcompensated by unusually large changes in entropy (ΔS = +100-120 J/mol·k) (Maeliche, et al., 1977). Detergent solubilized AcChR of Torpedo is known to have markedly different agonist binding properties when solubilized (Blanchard, et al., 1979).

Further kinetic results can be inferred from the observed temperature dependence. The denominator of Eq. 1 is the sum of two terms, T_{2b} and t_b. Because $1/t_b$ is the average exchange rate of the ligand-receptor complex, the data obtained from a

titration allows placement of a lower limit on the exchange rate. How close this limit is to the actual value depends on the contribution T_{2b} makes to the denominator. T_{2b} is not easily evaluated; however, the temperature dependence of the linewidth gives a good indication of the relative sizes of T_{2b} and t_b due to their opposite temperature dependences (Sykes, et al., 1970). The exchange rate ($1/t_b$) increases with temperature, exposing a larger proportion of the total choline spin population to the bound environment, and this results in a broadening of the observed line as temperature increases. T_{2b} is expected to have the opposite behavior because the faster molecular motions associated with higher temperature usually result in decreased effectiveness of relaxation processes and thus larger values for T_{2b} (Sykes, et al., 1970). A system in which T_{2b} was the dominant term in Eq. 1 would be expected to have a decreased linewidth at a higher temperature. The observation that the linewidth increased with temperature (Fig. 2) indicates that t_b is the dominant term, and, therefore, one may place meaningful lower limits on the dissociation rate of the agonist from the receptor complex. The dissociation rates determined (Table 1) are on the order of $2.5 \times 10^3 s^{-1}$ at room temperature. assuming a simple $L + R \rightleftharpoons LR$ mechanism, the lower limit of the association rate constant, k_{ass}, can be calculated from K_d and k_{diss} as $k_{ass} = k_{diss}/K_d = 1.5 \times 10^7\ M^{-1}\ s^{-1}$. Values in this range have been observed in stopped-flow experiments for binding a few agonists to membrane-bound (Grünhagen, et al., 1977;

Barrantes, 1978) to Torpedo AcChR as well as with Electrophorus electroplax in electrophysiological measurements (Sheridan and Lester, 1977). These values are on the threshold of being diffusion limited.

The following mechanism has been proposed for the mechanism of action of the partial agonist choline (Adams and Sackmann, 1978):

$$L + R \rightleftharpoons LR \rightleftharpoons LR^*$$

where LR* is the open channel form of the AcChR. This step is much less frequent when L is a partial agonist than is the case when L is an agonist such as acetylcholine. While this model is now known to be oversimplified (Dunn, et al., 1980), it is adequate to explain the NMR data if one takes into account the fact that choline is known (Quast, et al., 1978) to convert the AcChR into the closed channel high ligand affinity state thought to be an in vitro correlate of desensitization (Heidmann and Changeux, 1978). This is the state the AcChR was in under the equilibrium conditions of these NMR experiments, so that the observed mechanism was $L + R \rightleftharpoons LR'$, where LR' was the closed channel, high affinity state of the AcChR.

In conclusion, NMR has been shown to be a promising experimental method for investigating agonist binding to the purified AcChR in its membrane environment. Results that can be compared agree well with those obtained by using other methods, complications due to nonspecific binding effects or probe-induced

artifacts can be avoided, and the approach has provided a means of estimating parameters not previously determined.

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CHAPTER III

Agonist induced desensitization and its modulation by local anesthetics in highly purified membrane bound acetylcholine receptor

INTRODUCTION

After prolonged exposure to relatively low concentrations of agonist, the *in vivo* AcChR becomes electrophysiologically unresponsive to agonist concentrations that would normally generate a physiological response (Katz and Thesleff, 1957). The original investigators termed this phenomenon "desensitization" and proposed a two-state model which was capable of explaining their observations. The two-state model proposed that the AcChR has two affinity states for agonists and that the transition from low affinity to high affinity is mediated by the agonist itself. The high affinity state would correlate with a non-conducting form of the AcChR. Further studies (Rang and Ritter, 1969, 1970) found that this high affinity state is not ligand specific; desensitization induced by agonists increased the AcChR's affinity for certain antagonists as well.

The electrophysiological observations have been extended with *in vitro* studies of AcChR response to agonists. Agonist induced increase in ligand affinity has been detected (Weber, et al., 1975; Weiland, et al., 1976; Lee, et al., 1977) and this increase is thought to be an *in vitro* correlate of the desensitization observed electrophysiologically.

The *in vitro* observations could be qualitatively explained with a two state model (Weber, et al., 1975; Weiland, et al., 1976; Lee, et al., 1977), though quantitative agreement of the observations made in Ringer's buffer could not be achieved with a two-state model (Quast, et al., 1978). Rapid kinetics

studies using intrinsic AcChR fluorescence (Bonner, et al., 1976; Barrantes, 1976) and extrinsic probes (Grünhagen and Changeux, 1976; Quast, et al., 1978; Schimerlik, et al., 1979a,b) have been done to allow quantitative interpretation of conformational changes thought to represent desensitization. The studies performed with quinacrine (Grünhagen and Changeux, 1976) are complicated by the fact that quinacrine was in the same study shown to be a local anesthetic. Local anesthetics affect desensitization, as will be discussed below. Ethidium bromide was used in another kinetic study (Schimerlik, et al., 1979a,b; Quast, et al., 1978). This probe does not have local anesthetic properties and data collected using it would best be fit with a sequential binding model (Quast, et al., 1978, 1979).

Local anesthetics block the in vivo response of AcChR to agonists (Ruff, 1976; Neher and Steinbach, 1978), possibly by binding to the open channel form at the AcChR and blocking conductance. Local anesthetics also increase the rate of in vivo desensitization (Magazanik and Vyskocil, 1973).

The rate at which the affinity for agonists changes in vitro is also affected by the presence of local anesthetics. Dibucaine, for example, increases the rate of desensitization in vitro while tetracaine has the opposite effect (Blanchard, et al., 1979a). As in the case of in vivo desensitization, it is possible that the in vitro effects are due to direct interaction of the ligand with the protein; alternatively, there is the possibility that the effect is mediated by interaction of

the ligand with the lipid phase causing a change in AcChR conformation.

The nicotinic AcChR of the electric ray is composed of four types of subunits of 40,000, 50,000, 60,000 and 65,000 daltons (Weill, et al., 1974; Raftery, et al., 1974; Hucho, et al., 1978; Chang and Bock, 1979), although this composition is not universally held to be the case (Sobel, et al., 1977; Neubig, et al., 1979). Recently, however, the four subunits have been shown to all be part of the AcChR by amino acid sequence analysis (Raftery, et al., 1980). It has been shown that specific binding of a local anesthetic analog occurs on a separate polypeptide of 43,000 daltons (Sobel, et al., 1979; Blanchard and Raftery, 1979). This binding may in some way be associated with the AcChR and the 43,000 dalton protein has been the focus of much recent research (Neubig, et al., 1979; Changeux, et al., 1979; Moore, et al., 1979; Elliott, et al., (1979).

The recent observation (Neubig, et al., 1979; Elliott, et al., 1980) that a brief exposure of the AcChR enriched membrane fragments to high pH removes many extrinsic membrane proteins, including the 43,000 dalton polypeptide, has made it possible to further clarify the subunit composition of the membrane bound AcChR since only three (Neubig, et al., 1979) or all four (Elliott, et al., 1980) of the receptor subunits remain. Such alkali treated membrane fragments have been shown to possess the same activity as untreated membranes in terms of agonist mediated ion flux (Moore, et al., 1979), histrionicotoxin

binding (Elliott, et al., 1979), Carb binding (Dunn, et al., 1980), desensitization capability (Elliott, et al., 1979, 1980; Changeux, et al., 1979) and some aspects of local anesthetic modulation of the desensitization rate in that the local anesthetic trimethisoquin was found (Changeux, et al., 1979) to increase the rate of desensitization. The question still remains whether or not the different types of modulation elicited by the different local anesthetics are retained in alkali treated membranes containing only the AcChR protein. It has been demonstrated that several different local anesthetics displace specifically bound [^{3}H]-H_{12}-HTX (Elliott, et al., 1979; Blanchard, et al., 1979) and that this toxin binds specifically to the AcChR (Elliott and Raftery, 1979; Elliott, et al., 1979; Neubig, et al., 1979, Eldefrawi, et al., 1977) rather than to proteins distinct from the AcChR (Sobel, et al., 1978; Eldefrawi, et al., 1979). However, it has not been shown that the binding of the various agents is a strictly competitive process and the possibility remains that some local anesthetics that modulate the AcChR's affinity for agonists exert their effects by conformationally linked processes involving entities other than the AcChR.

I report here that membrane fragments containing only the AcChR protein complex exhibit both positive and negative modulation of the rate of agonist induced ligand affinity in the presence of dibucaine and tetracaine, respectively.

MATERIALS AND METHODS

I. AcChR Enriched Membrane Fragments

Approximately 500 g of frozen electric organ from T. californica was broken into small pieces and mixed with 500 ml of buffer [10 mM NaPi, 5 mM EDTA, 0.02% NaN_3, 5 mM iodoacetimide, 10 mM PMSF pH = 7.4]. This was ground for 2 minutes in a Waring blender, then reground for 4 x 30 seconds at 30,000 rpm in a Virtis 60 homogenizer. Large particles and connective tissue were removed by a 10 minute centrifugation at 5000 rpm in a Sorval GSA rotor. The supernatant was then centrifuged 60 minutes at 30,000 rpm in a Beckman 35 rotor. The pellet was resuspended in 10 mM NaPi (pH 7.4) 1 mM EDTA by using 2 x 30 second homogenizations with a Virtis 60 homogenizer. Sucrose and NaCl were added to achieve final concentrations of 30% (w/w) and 0.4 M, respectively.

The sample (10 ml) was layered on a discontinuous sucrose gradient with steps of 5 ml 50% w/w, 5 ml 39% w/w, and 12 ml of 30% w/w, all in 0.4 M NaCl, 10 mM Pi (pH = 7.4) 1 mM EDTA. This buffer was used to adjust the final volume of each tube to the rim. The loaded tubes were centrifuged for 60 minutes at 45,000 rpm in a VTi 50 rotor using a slow acceleration accessory and Beckman L-5 centrifuge. After centrifugation the membrane fragments were observed to be in three bands. The middle band, consisting of particles with a density of 1.18 g/cc, was aspirated away from the gradients, diluted with plain buffer and centrifuged for 60 min at 35,000 RPM in

a Beckman 35 rotor. The membranes were resuspended in a small amount (∿10 ml) of Torpedo Ringer's buffer using a Virtis homogenizer. This preparation has been described in detail in Elliott, et al., 1980).

II. Alkali Treatment of AcChR Enriched Membrane Fragments

Alkali treatment of the membrane fragments from the above procedure was adapted from the protocol reported by Steck and Yu (1973) for erythrocyte ghosts. Membrane fragments containing approximately 50 mg of total protein were pelleted, resuspended in distilled water with a Virtis homogenizer and pelleted again. They were then resuspended in 80 ml of cold distilled water using a Virtis homogenizer for 2 x 30 sec. The suspension was then adjusted to pH 11.0 using 0.5% NaOH. This pH was maintained for 30 minutes at 4°, with small additions of NaOH. The membranes were then pelleted by centrifugation for 30 minutes at 30,000 rpm in a Beckman type 35 rotor. A Virtis homogenizer was used to resuspend the pellet in Torpedo Ringer's buffer.

III. Affinity-State Assay

The state of affinity of the AcChR for the agonist Carb was determined by a competitive binding assay (Quast, et al., 1978). The rate of α-BuTx-AcChR complex formation was determined by adding 2.6 x 10^{-7}M [^{125}I]-α-BuTx to 5 x 10^{-7}M AcChR in Ringer's buffer and pipetting 50 μL of this solution onto 2.4 cm Whatman DE-81 filter paper discs at given time intervals, after which the discs were washed and their radioactivity

measured in a γ-counter. This was repeated with an identical solution which had been preincubated for 30 minutes with 1 μM Carb to insure that all AcChR was in the high affinity state. Having established the two control rates, the toxin on-rate was measured when Carb was added simultaneously with α-BuTx. The total AcChR population is a mixture of receptors of either high or low affinity, and thus the observed rate of α-BuTx-AcChR complex formation is composed of two components; one in which α-BuTx competes with Carb for receptors with low Carb affinity, and a slower rate in which toxin competes with Carb for those receptors having the higher (∿1000 X) agonist affinity. The closer the net rate is to the previously measured toxin on-rate in the absence of Carb, the greater the proportion of the receptor population in the low agonist affinity state. Experiments in which the rate of the agonist-induced affinity state transition were measured were performed only with preparations in which the majority of receptors were in the state of low affinity for agonists.

The rate at which agonists induced the affinity state change is affected by some local anesthetics and this effect was quantitatively assayed by a modification of the affinity state assay. The appropriate local anesthetic was added to the AcChR in Ringer's buffer and after a minimum of 30 minutes, α-BuTx Carb competition assays were performed with various Carb preincubation times, allowing the transition rate from low to high affinity to be monitored. The rate was calculated from

these data using the equation derived by Blanchard, et al. (1979a);

$$K_{(app)} = (k_o - k_\infty)\, e^{-k't} + k_\infty$$

where k_o was the rate of toxin binding in the presence of 1 μM Carb without preincubation, k_∞ was this rate in 1 μM Carb when all AcChR was in the high affinity state (30 minutes preincubation) and k' was the rate of transition in AcChR affinity to agonist.

IV. Miscellaneous

AcChR concentration was determined by complexing the AcChRs with saturating amounts of [^{125}I]-α-BuTx and adsorbing these complexes to Whatman DE-81 DEAE paper discs. Uncomplexed toxin, which has a positive charge, was removed by washing the discs in 10 mM phosphate (pH 7.4) 100 mM NaCl buffer with 0.1% Triton X-100. The washed discs were then assayed for radioactivity (Schmidt and Raftery, 1973). Protein concentrations were determined using the method of Lowry, et al. (1951). SDS polyacrylamide gels were done according to the method of Laemmli (1970).

RESULTS

The membranes purified by sucrose gradient centrifugation had a complex polypeptide composition as evidenced by SDS gel electrophoresis and had a specific activity of ∿1 nmole [^{125}I]-α-BuTx binding sites per mg protein. Following alkali treatment the resultant membranes consisted of only four principal proteins staining intensely with CBB and migrating as 40, 50, 60 and 65 x 10^3 dalton components on gel electrophoresis (Fig. 1). The specific activity of these membrane preparations was typically ∿3 nmoles [^{125}I]-α-BuTx bound per mg protein, a value close to that previously reported (Elliott, et al., 1980). The four polypeptides are those previously reported for detergent solubilized AcChR purified by affinity chromatography (Raftery, et al., 1974; Weill, et al., 1974; Hucho, et al., 1978; Chang and Bock, 1979; Deutsch and Raftery, 1979) from Torpedo californica and other related species (Deutsch and Raftery, 1979). The supernatant from the alkali treatment contained principally a 43,000 dalton polypeptide and the soft pellet a 90,000 dalton polypeptide, both major components of the membranes before the base extraction (Fig. 1). Thus the hard pellet represents a membrane fraction essentially devoid of these two major components as well as other minor contaminants and is composed almost entirely of the four AcChR subunits. The polypeptide composition of this preparation is not universally agreed upon since others have reported that it contains three, not four, of the AcChR subunits (Neubig,

et al., 1979; Changeux, et al., 1979), i.e., those of 40, 50 and 65 x 10^3 daltons. Based on the recent finding that all four of the AcChR subunits are homologous polypeptides (Raftery, et al., 1980) it was concluded that all are part of a pentameric receptor complex with a stoichiometry of 2:1:1:1 for the subunits of 40, 50, 60 and 65 x 10^3 daltons, respectively (Strader, et al., 1980) and that the 60,000 dalton polypeptide is not a contaminant in the preparations used here.

The alkali extracted membranes underwent a Carb mediated affinity state change in a manner similar to the untreated membranes. This was demonstrated using the effects of Carb on the time course of [^{125}I]-α-BuTx-AcChR complex formation (Fig. 2). Prolonged exposure to cholinergic ligand caused an increase in the affinity of the AcChR for the ligand with a consequent increase in its effectiveness as an inhibitor of [^{125}I]-α-BuTx binding. The final experimental point was taken 24 hours after the reactions were initiated and it was shown that the amount of [^{125}I]-α-BuTx bound at equilibrium was unchanged by the presence of Carb. A plot of the log of the difference between the amount of [^{125}I]-α-BuTx bound at each time interval and at equilibrium versus time was linear over the total time interval measured. The pseudo-first order rate constants derived from these data are given in Table 1.

The effects of two local anesthetics on the rate of the Carb mediated affinity state change were next investigated. The anesthetics chosen (dibucaine and tetracaine) were those

that previously have been shown to have opposite effects on this rate in AcChR enriched membrane preparations that, however, still contained the contaminating polypeptides of 43 and 90 x 10^3 daltons as well as many minor protein components. It was therefore not possible to state whether the effects observed on the rate of the Carb mediated affinity change were due to specific association of the local anesthetics with the AcChR or with other protein components of the membrane.

In the studies described here the basic affinity state assay procedure was modified to include a 30 minute preincubation of membranes with the local anesthetic prior to addition of [^{125}I]-α-BuTx, [^{125}I]-α-BuTx plus 1 μM Carb to start the reaction. As shown in Figure 3, incubation with dibucaine (1 μM) dramatically enhanced the Carb induced rate of transition of the AcChR from a low to a high affinity state.

Using another common local anesthetic, tetracaine, I found (Fig. 3) that, as in the case of membranes not treated with alkali, it decreased the rate of Carb mediated conversion of affinity states. Thus the positive and negative modulating effects of dibucaine and tetracaine on the conversion are retained in membranes that contain only the AcChR protein and are devoid of a different polypeptide (43 x 10^3 daltons) that has previously been shown to interact with local anesthetics (Sobel, et al., 1979; Blanchard and Raftery, 1979).

DISCUSSION

The magnitude of the effect of both anesthetics on the Carb mediated transition was similar for the alkali treated preparation used in the studies reported here and for the untracted preparations described earlier (Blanchard, et al., 1979b). Since less than 2% of the contaminating polypeptides of 43 and 90 x 10^3 daltons remained in the base treated preparation the results strongly support the notion that the site(s) to which local anesthetics bind and exert their modulating effects on AcChR conformational transitions induced by agonists represent binding sites on the receptor macromolecular complex.

Tetracaine was found to decrease the rate of agonist induced desensitization, which is the opposite of what was reported by Weiland, et al., (1977) for this compound. However, the latter study used a buffer which did not contain calcium and hence was not representative of physiological conditions. Calcium has been shown to increase that rate of desensitization *in vitro* (Lee, et al., 1977), hence the studies are not directly comparable.

Dibucaine increased the rate of desensitization, in keeping with a recent report (Changeux, et al., 1979) which also described a similar effect with regard to the local anesthetic dimethisoquin causing enhancement of the rate of a process caused by a fluorescent agonist binding to AcChR enriched membranes prepared from *Torpedo marmorata*.

Interestingly, the direction of the effects of dibucaine and tetracaine matches their affinities for the high and low affinity states of the AcChR site as measured by [^{3}H]-H_{12}-HTX binding, an observation first made by Blanchard, et al. (1979a). Tetracaine binds relatively more tightly to the low affinity form of AcChR and also slows the rate of desensitization. Dibucaine increases the rate of desensitization and binds relatively more tightly to the high affinity form of the AcChR. These authors proposed that the magnitude and direction and a local anesthetic's effects on the rate of desensitization may be predicted by the ratio of its K_Is for the HTX binding site in the high and low affinity AcChR states.

Table I

Rates of carbamylcholine induced transition from low agonist affinity to high agonist affinity.

	Membranes	Alkali Treated Membranes
Carb only	$.002 \pm .001 \ s^{-1}$	$.002 \pm .0005 \ s^{-1}$
+Dibucaine	$.015 \pm .002 \ s^{-1}$	$.010 \pm .001 \ s^{-1}$
+Tetracaine	$.0005 \pm .0002 \ s^{-1}$	$.0008 \pm .0002 \ s^{-1}$

Figure 1. SDS polyacrylamide gel scans:

A. Membrane fragments

B. Alkali treated membrane fragments

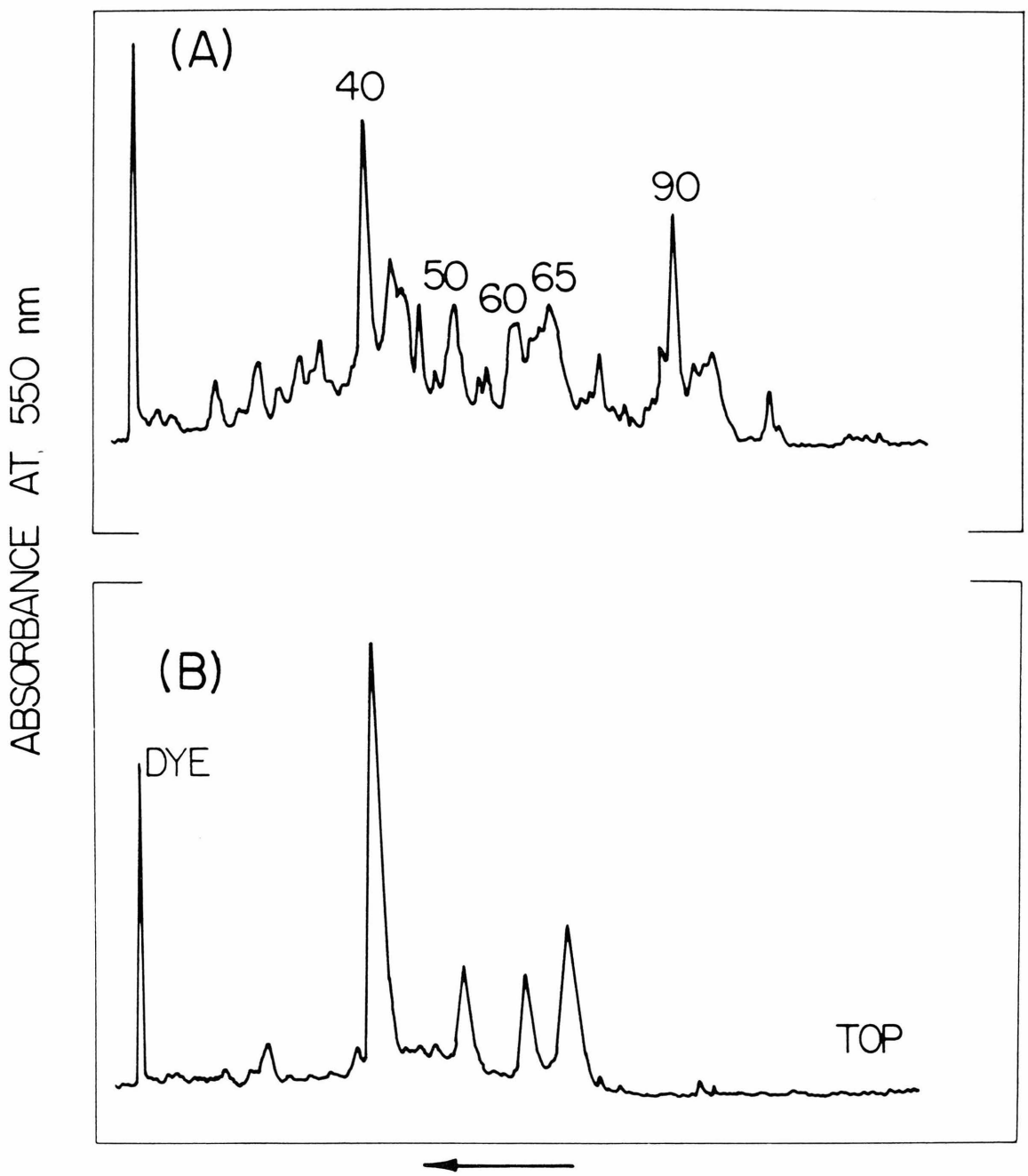

(A)
40
50
60
65
90
(B)
DYE
TOP
ABSORBANCE AT 550 nm

Figure 2. Assays of the affinity state of the acetylcholine receptor for carbamylcholine.

A) As described in Materials and Methods, membrane fragments were assayed for their relative carbamylcholine affinity. The plot is of log $(X_\infty - X_t)$ vs time, where X_∞ is the amount of α-BuTx bound at equilibrium and X_t is the amount of α-BuTx bound at the specified point. The TOP LINE represents [^{125}I]-α-BuTx binding without presence of carbamylcholine, the MIDDLE LINE represents [^{125}I]-α-BuTx binding when 10^{-6} M carbamylcholine is added simultaneously with the [^{125}I]-α-BuTx, and the BOTTOM LINE represents [^{125}I]-α-BuTx binding to the acetylcholine receptor which has been desensitized by a 30 minute preincubation with 10^{-6} M carbamylcholine.

B) The affinity state assay described above using alkali treated membrane fragments.

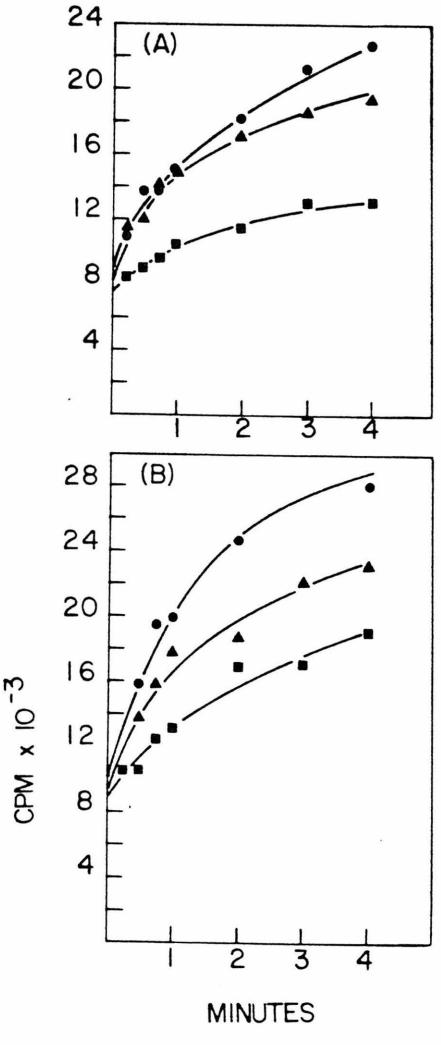
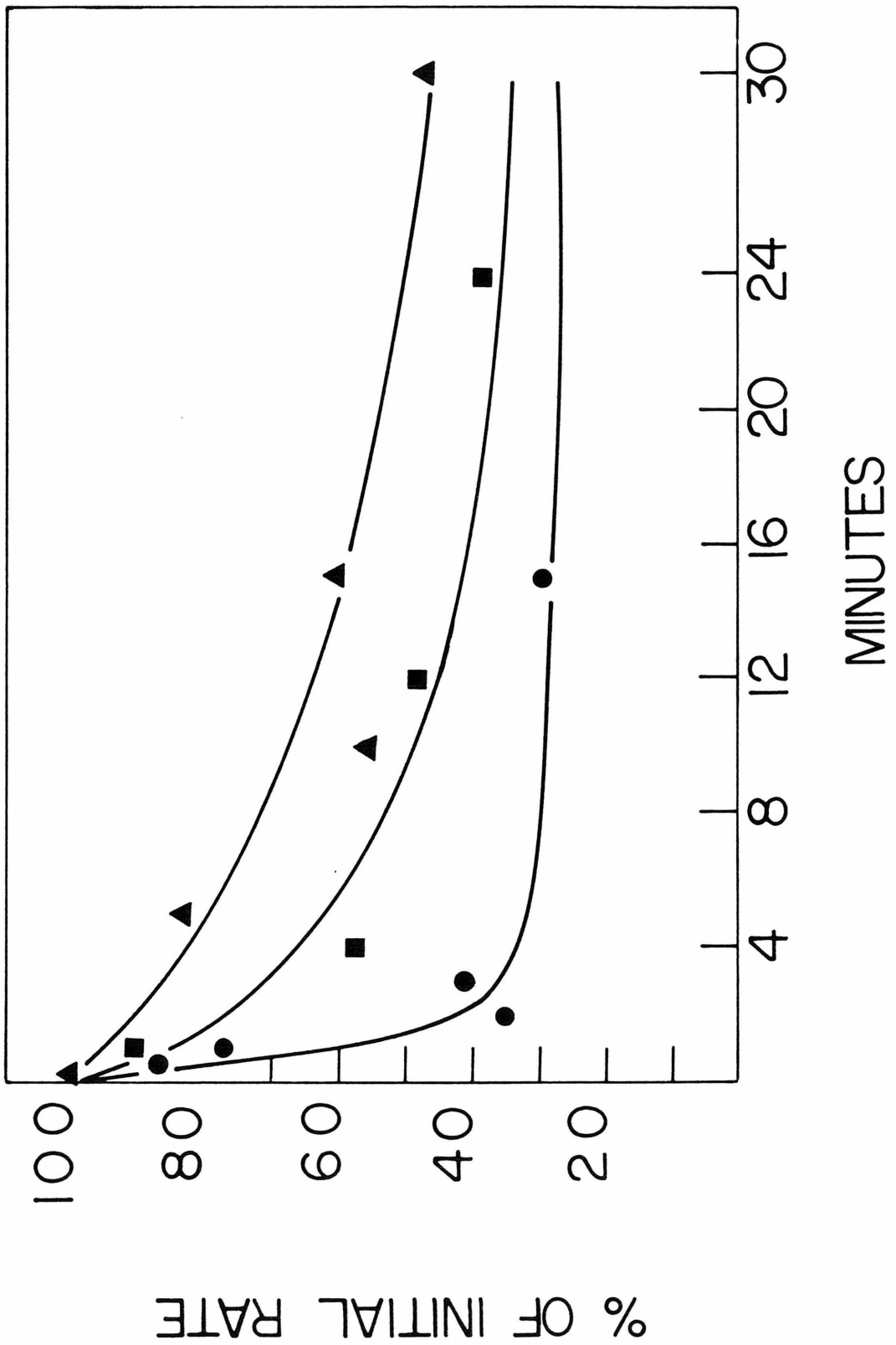

(A)
(B)
24
20
16
12
8
4
28
1
2
3
4
CPM x 10^{-3}
MINUTES

Figure 3. Change in the rate of carbamylcholine induced affinity change in the presence of local anesthetics. Membrane fragments which had been treated were incubated 30 minutes with 50 μM tetracaine (▲-▲), without local anesthetic (■-■), or with 1 μM dibucaine (●-●). 10^{-6} M carbamylcholine was added and the membranes incubated for the indicated times before [^{125}I]-α-BuTx was added and the rate of complex formation assayed as described in Materials and Methods. The plots show the apparent rate of toxin binding vs time of incubation with carbamylcholine. The rate constants for the transitions were calculated as described in the Methods section and are listed in Table 1.


% OF INITIAL RATE
100
80
60
40
20
4
8
12
16
20
24
30
MINUTES

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