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STUDIES OF THE INTERACTION OF AGONIST, ANTAGONIST AND ION  
CONDUCTANCE CHANNEL BINDING SITES IN THE FUNCTIONAL  
ACETYLCHOLINE RECEPTOR COMPLEX FROM FROG  
NEUROMUSCULAR JUNCTION

*City University of New York*

PH.D. 1982

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STUDIES OF THE INTERACTION OF AGONIST, ANTAGONIST  
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IN THE FUNCTIONAL ACETYLCHOLINE RECEPTOR COMPLEX  
FROM FROG NEUROMUSCULAR JUNCTION.

by

STEVEN GROSS

A dissertation submitted to the Graduate Faculty in Pharmacology  
of the School of Biomedical Sciences in partial fulfillment of the  
requirements for the degree of Doctor of Philosophy, The City  
University of New York.

1982



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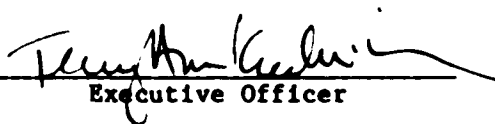
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This manuscript has been read and accepted for the Graduate Faculty in Pharmacology of the School of Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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## LIST OF ABBREVIATIONS

The following abbreviations have been employed to indicate the names of molecular species which occur repeatedly throughout this text.

nAChR = nicotinic acetylcholine receptor complex

$\alpha$ -BTX =  $\alpha$ -bungarotoxin

dTC = d-tubocurarine

PCP = phencyclidine

DPA = N, N, dimethyl phenylaziridinium

DTT = dithiothreitol

## ABSTRACT

This study attempts to determine allosteric changes in the acetylcholine receptor ion channel complex (nAChR) of frog neuromuscular junction that may be induced by the binding of small ligands prototypic of each of three functional classes, i.e., agonists (carbachol), antagonists (d-tubocurarine), and ion channel blockers (phencyclidine). The study characterizes and makes use of the compound N, N, dimethyl phenylaziridinium (DPA) which is shown to behave as an irreversible agonist in dithiothreitol (DTT) reduced frog muscle. Changes in the binding characteristics of [ $^{125}$ I]  $\alpha$ -bungarotoxin ( $\alpha$ -BTX) were employed as a specific probe to detect for the conformational changes of the receptor. The major findings of this study are summarized below.

1. The actions of DPA following DTT reduction of rectus abdominis muscle are consistent with that of an irreversible (alkylating) nicotinic agonist.
  - A. The DPA response is prevented by pretreatment with BTX ( $2 \times 10^{-7}$  M) or with dTC ( $10^{-6}$  M).
  - B. DPA induced contracture of reduced muscle is protected by carbachol ( $10^{-5}$  M) and dTC ( $10^{-6}$  M).
  - C. DPA induced contracture is reverted by the channel blocking ligands PCP (25  $\mu$ M) and adiphenine (25  $\mu$ M).
  - D. In common with carbachol the initial tension response to DPA is diminished by muscle reduction with DTT.
2. A "ternary" complex is formed with nAChR,  $\alpha$ -BTX and DPA.
  - A. DPA alkylation results in no reduction in the number of binding sites for  $\alpha$ -BTX, however, it greatly reduces the affinity of  $\alpha$ -BTX for the receptor indicating a change in conformational

state of the binding protein.

- B.  $\alpha$ -BTX ( $2 \times 10^{-7}$  M) reverts the contracture in DPA alkylated muscle indicating simultaneous occupancy.

3. The agonist and antagonist binding sites are distinct in intact muscle nAChR.

The ability of carbachol but not dTC to compete for [ $^{125}$ I]  $\alpha$ -BTX binding to whole sartorius muscle is greatly diminished by DPA alkylation.

4. The binding of a channel binding ligand may influence the conformation of the ACh recognition site.

PCP (25  $\mu$ M) protects against DPA alkylation, however, at this concentration PCP has no effect on  $\alpha$ -BTX binding.

5. The binding of agonist influences the conformation of the binding site for PCP on the ion conductance channel.

PCP binds with higher affinity to the agonist activated than to the resting (non-conducting) conformation of the channel.

6. Extraction of the nAChR from its membrane environment results in drastic changes in ligand binding properties and/or the ability of a ligand to induce conformational changes.

- A. Whereas  $\alpha$ -BTX binding data to membrane bound nAChR can be explained by a single kinetic class of binding site, solubilization results in an equal number of two kinetically distinct classes (20-fold different in affinity).

- B. Whereas in intact muscle DPA alkylation reduces the affinity but not number of  $\alpha$ -BTX sites, in extracted nAChR DPA alkylation reduces number but not affinity of sites.

- C. Qualitative effects are observed in the ability of carbachol

and dTC to differentially effect  $\alpha$ -BTX binding in soluble and membrane bound nAChR.

## INTRODUCTION

### 1. The Acetylcholine Receptor Complex - Some General Properties

Skeletal muscle contraction is initiated by the interaction of acetylcholine (ACh), released by nerve endings, with a receptor protein which is an integral component of the muscle cell membrane. This receptor (AChR) was classified as the nicotinic acetylcholine receptor (nAChR) in terms of the spectrum of agents which mimic the action of ACh (agonists) or inhibit its activity (antagonists) (Dale, 1914). In normal adult muscle nAChR is localized to the small specialized area of sarcolemmal membrane (termed end plate) in close apposition to the motor nerve ending (Barnard et al., 1971; Fambrough and Hartzell, 1972; Salpeter and Eldefrawi, 1973). Released ACh combines with distinct loci on the membrane-bound nAChR (the recognition site) which induces a local increase in cation permeability through a specifically related ion conductance channel (ICC). This results in an increase in conductance from essentially zero to about  $2-3 \times 10^{-11}$  mho (Stevens, 1974). The increased conductance at the end plate area results in a net influx of positive charge and a limited region of membrane depolarization i.e. the end plate potential (EPP). The EPP, which is caused by the summation of current flow through individual receptor linked ion channels, may give rise to an action potential (in most skeletal muscle) and muscle contraction if the EPP depolarization reaches the threshold level required to initiate the all-or-none propagated action potential in the sarcolemmal membrane.

The nAChR is seen to serve two functions, that of signal recognition in response to ACh and that of mediating the permeability response to the signal. As a result, the functional nAChR can be con-

sidered to be composed of two distinct components; a recognition protein which has binding sites for agonists and antagonists (i.e. ACh, tubocurarine (dTC), snake venom toxins) and an ion conductance component which is linked, at least functionally, to the recognition site.

Although some evidence suggested that the two components are distinct and separable proteins (Eldefrawi, 1979; Sobel and Changeux, 1979) more recent reconstitution experiments indicate that the two functional components may be contained in a single multiple polypeptide protein which represents the receptor complex (Changeux, 1980; Raftery et al., 1980). The ICC component appears capable of binding a distinct group of drugs (i.e. local anesthetics (Krodel et al., 1979; Neher and Steinbach, 1978), histrionicotoxin (Eldefrawi et al., 1977), and phencyclidine (Albuquerque et al., 1980)) which alter the time course and amplitude of the passage of ions through the channel and thereby affect the characteristics of the end plate current. These drugs are thought not to bind directly to the recognition component in the concentration range of their biological activity. They may, however, behave as allosteric effectors (Monod et al., 1965) to the recognition component. Additionally, some drugs may have binding sites on both the ICC and recognition components of the receptor. Although agonists and antagonists have been generally considered as competitors for a single binding site on the recognition protein, a mutual interaction between distinct (or partially overlapping) binding sites for each of these two classes of ligand may not be ruled out. Thus there is the possibility of at least three distinct drug binding sites on the receptor complex.

A large body of evidence suggests that the signal function of the nAChR is dependent on ligand-induced transitions between at least three

discrete conformational states (Changeux et al., 1976; Heidmann and Changeux, 1978). The resting conformation (R) in which channels are in a non-conducting (closed) state undergoes a rapid conversion when agonist is bound to an "active" (open) channel-ion conducting conformation (A). This conformational transition was first proposed by Nachmansohn at a time when receptors were purely hypothetical (Nachmansohn, 1955). Prolonged or repetitive agonist treatment causes receptors in the A conformation to slowly convert to a desensitized state (D) associated with closed channels or channels with limited maximum conduction (partially open?). Although the "D" conformation binds agonist ligands it is refractory to agonist activation, that is, the conformation "A" cannot be realized by a conformational transition from the "D" state. This corresponds to the well known phenomenon of pharmacological desensitization (Katz and Thesleff, 1957). Evidence has been presented that ACh and other cholinergic ligands may possess different affinities for each of the nAChR conformational states in membrane bound receptor preparations (Heidmann and Changeux, 1978). These changes in affinity could thus provide an experimental measure to detect such changes in receptor conformation.

## 2. Vertebrate muscle nAChR-Recognition Protein

A. Factors facilitating study - Rationale for the use of  $\alpha$ -BTX as a receptor probe.

In the past ten years a large body of information has been obtained from studies on the anatomy, physiology, and biochemistry of the nicotinic AChR. As a result it is by far the best understood of all neurotransmitter receptor proteins. The productivity of research in this area is based largely in two fortuitous findings. The first finding,

that the specialized electric organ (electroplax) from electric fish, *Electrophorus* and torpedo (eel and ray), is a tissue enriched about a thousand fold (compared to striated muscle) in molecules involved in cholinergic transmission (Feldberg and Fressard, 1942; Keynes and Martins-Ferreira, 1953) has provided researchers with a receptor in sufficient quantities for detailed study. Secondly, it has been demonstrated that the long known curare-like action of certain neurotoxins derived from the venoms of elapid (cobra, mamba and krait) and hydrophid (sea snakes) (Chang and Lee, 1963; Chang and Lee, 1966) is attributable to their high affinity and specificity for electroplax and vertebrate muscle nAChR. These toxins (i.e.  $\alpha$ -Bungarotoxin ( $\alpha$ -BTX), cobratoxin), as their radioactive derivatives, have proved to be invaluable tools for the quantitation, characterization, and purification of the nAChR. The work reported here utilizes  $\alpha$ -BTX and its moniodo radioactive derivative [ $^{125}$ I]  $\alpha$ -BTX as a quantitative probe for the cholinergic receptor from vertebrate skeletal muscle.

$\alpha$ -BTX was isolated and characterized from the venom of the Malayan banded Krait, *Bungarus multicinctus*, by Chang and Lee (Chang and Lee, 1963). It was found to be a basic polypeptide composed of 74 amino acids in which a region of homology in common with other  $\alpha$ -neurotoxins is observed (thr-25, trp-29, lys-27 and arg-33). The toxin has a very slow dissociation rate from the nAChR and produces a practically irreversible non-depolarizing blockade at the postsynaptic membrane of all vertebrate neuromuscular junctions studied (i.e. frog (Chang and Lee, 1963; Miledi and Potter, 1971); chick (Chang and Lee, 1963; Su et al., 1967); mouse (Chiu et al., 1974); rat (Albuquerque et al., 1973)). The essentially irreversible nature of the blockade as compared

with other elapid toxins of this type (i.e. cobratoxin) is thought to derive from the larger number of lipophilic amino acids in  $\alpha$ -BTX (Lee, 1972). In frog muscle  $\alpha$ -BTX inhibits the response elicited by iontophoretically applied ACh (Miledi and Potter, 1971) but is without effect on KCl induced contraction (Chang and Lee, 1963). It inhibits end plate potentials (EPPs) as well as miniature end plate potentials (MEPPs), but has no effect on conduction or depolarization in the nerve terminal or on passive electrical properties of the muscle membrane (Lee, 1972).  $\alpha$ -BTX shows no demonstrable inhibition of ACh release from nerve endings (Chang and Lee, 1963). Finally, reversible cholinergic ligands at low concentration, both agonists and antagonists (20  $\mu$ M carb or decamethonium), can protect completely from a concentration of  $\alpha$ -BTX (5  $\mu$ g/ml) which would otherwise cause irreversible inhibition of twitch tension in mouse diaphragm (Sarvey et al., 1978). Thus, taken together, these physiological findings strongly suggest a mode of neuromuscular block that is essentially identical to curare with the exception that  $\alpha$ -BTX is much more slowly dissociable from the nAChR.

Autoradiographic localization of  $\alpha$ -BTX binding in skeletal muscle is in excellent agreement with the known distribution of acetylcholine receptors. The toxin molecules are readily labelled with radioactive iodine allowing visualization and quantitation of nAChR at the fmole level by autoradiography (Lee and Tseng, 1966). An accumulation of labelled  $\alpha$ -BTX has been observed at the motor end plate of mouse and rat diaphragm (Lee and Tseng, 1966; Porter et al., 1973). Both the binding and inhibition of response could be prevented if reversible cholinergic ligands were incubated with tissue simultaneously with labelled toxin. The distribution of grain densities about the synapse indicates  $\alpha$ -BTX

6  
binding has a predominantly (>90%) postsynaptic as opposed to a presynaptic location (Porter et al., 1973).

It is well established that denervation of muscle induces a large increase in sensitivity to iontophoretically applied ACh as well as a spread in ACh sensitivity to non-end plate areas (Miledi, 1960). After denervation of rat diaphragm a 20-fold increase in toxin binding is observed relative to non-denervated control muscles (Miledi and Potter, 1971; Lee et al., 1967; Hartzell and Fambrough, 1973). An excellent correlation between autoradiographic appearance of  $\alpha$ -BTX sites and the time course of sensitivity increase to applied ACh following denervation has been observed (Hartzell and Fambrough, 1973; Lee et al., 1967). Both effects were shown to have a similar post-denervation lag period and time to peak response (14 days).

Acetylcholine responsiveness has been studied in embryonic muscle developing in tissue culture (Hartzell and Fambrough, 1973; Sytkowski et al., 1973; Vogel et al., 1972). Mononucleated myoblasts do not exhibit a depolarization in response to iontophoretically applied ACh and likewise show no significant binding of [ $^{125}$ I]  $\alpha$ -BTX. Sensitivity to ACh appears after several days in culture when myoblasts fuse to form multinucleated myotubes. The binding of  $\alpha$ -BTX and its ability to block ACh induced depolarization develop in parallel. Initially,  $\alpha$ -BTX binding sites are observed to be distributed homogeneously on the myotube membrane, however, they later begin to form clusters in which the density has been measured to be  $9,000/\mu\text{M}^2$  (Sytkowski et al., 1973) similar to that measured at the adult motor end plate (Porter et al., 1973). In summary, receptor site density as judged electrophysiologically from ACh induced depolarization has been successfully predicted

from analysis of labelled  $\alpha$ -BTX binding; indicative of a highly specific interaction between  $\alpha$ -BTX and the nAChR.

Owing to the rich concentration, the great majority of biochemical and biophysical studies have employed nAChR derived from electroplax. While vertebrate skeletal muscle contains only 1-4 picomoles of  $\alpha$ -BTX binding sites/gram tissue (Miledi and Potter, 1971; Colquhoun et al., 1974; Berg et al., 1972), torpedo and electrophorus electroplax have been measured to contain 1000 picomoles/gram (Miledi et al., 1971) and 20-30 picomoles/gram (Rafferty et al., 1971), respectively. Electroplax are derived embryonically from skeletal muscle and contains postsynaptic receptors having a nicotinic pharmacology (Keynes and Martins-Ferreira, 1953). In common with skeletal muscle receptors, electroplax receptors show an approximately equal increase in permeability to sodium and potassium ions (Ruiz-Manresa and Grundfest, 1971) in response to agonist activation. The response to ACh in electroplax is inhibited competitively by dTC and is also inhibited by the snake venom  $\alpha$ -neurotoxins (Lee, 1972). Immunological experiments provide further support for the contention that mammalian receptors are structurally very similar to electroplax receptors as evidenced by the fact that rabbits immunized with highly purified electrophorus receptor develop an extreme muscular weakness. Antibodies raised to torpedo nAChR cross react with rabbit skeletal muscle nAChRs (Patrick and Lindstrom, 1973). Each of the five glycopeptide subunits which compose the torpedo receptor has been demonstrated to have some antigenic determinants in common or closely homologous with the subunits of mammalian muscle receptor (Lindstrom et al., 1979). Although the mammalian receptor subunit structure appears to be similar to that isolated from

electroplax, distinct differences in site directed alkylation have been observed (Froehner et al., 1977; Nathanson and Hall, 1979).

#### B. Localization and membrane integration

The localization of the "receptive substance" for nicotine was first described by Langley (Langley, 1907). He simply applied the nicotine dropwise along the length of frog sartorius muscle fibers and found that maximal response is achieved at the end plate region. Langley's observation of the localization of agonist sensitivity to the neuromuscular junction in normal skeletal muscle has been upheld and greatly refined with the development of more sophisticated techniques (i.e. microiontophoresis, voltage clamping). Kuffler et al. (1975) have shown that the sensitivity of the sarcolemmal membrane only 2 micrometers away from the synapse is 50 times lower than that of the subsynaptic membrane to iontophoretically applied ACh. At a distance of 200 micrometers from the synapse the sensitivity diminishes to only 1/1,000 that of the endplate value.

Electron micrographic studies utilizing peroxidase conjugated  $\alpha$ -BTX have demonstrated that  $\alpha$ -BTX binding sites localized to the end plate region are concentrated in the crests of junctional folds in regions of thickened membrane which comprise approximately 50% of the end plate area (Fertuck and Salpeter, 1974; Daniels and Vogel, 1975; Albuquerque et al., 1974). Earlier estimates of the nAChR site density in mouse motor end plate (Porter et al., 1973), judged from autoradiographic distribution of  $\alpha$ -BTX binding have been recalculated utilizing this information to give a value of 30,000 sites/micrometer<sup>2</sup> ( $\pm 30\%$ ) for the receptor rich crests of the junctional folds (Fertuck and Salpeter, 1976). Receptor site density is found to diminish to 50 toxin

sites/micrometer<sup>2</sup> at 15 micrometers away from the end plate and is only 10-20 toxin sites/micrometer<sup>2</sup> in extrajunctional regions. These results are in excellent agreement with the receptor localization as determined by cholinergic agonist sensitivity.

Diffuse distribution of nAChR has been observed in both embryonic (Diamond and Miledi, 1962) and denervated skeletal muscle (Axelson and Thesleff, 1959). Although these forms of receptor appear to be very similar biochemically to "normal" endplate nAChR, physicochemical (Brookes and Hall, 1975) and immunological differences (Almon and Appel, 1976) have been reported. Also differences in receptor lifetime (Berg and Hall, 1974; Chang and Huang, 1975) and pharmacological sensitivity (Chiu et al., 1974; Beranek and Vyskocil, 1967) have been observed.

Electron microscopy of receptor enriched electroplax membranes which have been negatively stained with sodium phosphotungstate reveals a regular arrangement of 80-90 Å particles (Cartaud et al., 1978; Ross et al., 1977). Each of these particles appears to be a rosette composed of 5-6 subunits which have a diameter of 30-40 Å and surround a central pit filled by the electron dense stain. The rosettes appear to be arranged in regular-hexagonally packed arrays with an inter-particle distance of 90-100 Å. These particles have often been interpreted to be individual nAChR complexes. The major support for this view is the observation of virtually identical rosette-like particles in negatively stained detergent micelles which contain purified electroplax nAChR (Cartaud et al., 1978; Ross et al., 1977; Meunier et al., 1974). The particle density in torpedo receptor rich membranes has been measured to be 12,000-15,000 per micrometer<sup>2</sup> (Cartaud et al., 1978) whereas the α-BTX site density in electroplax subsynaptic membranes has been reported

to be  $50,000 \pm 16,000/\text{micrometer}^2$  (Bourgeois et al., 1978). If the particles do indeed represent the morphological counterpart of the nAChR then each rosette should contain 2-4  $\alpha$ -BTX binding sites. This stoichiometry for toxin binding sites is consistent with results obtained using purified nAChR.

Freeze fracture of neuromuscular membranes from a variety of animal species displays intramembranous particles having a repetitive arrangement in areas where nAChR density is expected to be high (Heuser et al., 1974). In torpedo membranes which have been subjected to freeze fracture  $80 \text{ \AA}$  particles are observed to occur most frequently fixed to the cytoplasmic leaflet (A face) in an outward direction (Changeux et al., 1976). They are arranged in geometric arrays having a center to center distance of 90-100  $\text{\AA}$ . The inwardly directed membrane surface (B face) bears depressions complementary to the A face particles, as well as occasional  $80 \text{ \AA}$  particles. These findings are in agreement with the notion that the nAChR is an integral protein which spans the width of the membrane and is exposed on both surfaces.

C. Muscle nAChR recognition protein - Isolation, purification and characterization.

Although much valuable knowledge has been derived from studies of the biochemistry of electric fish nAChR, the application of this knowledge to our understanding of the mammalian neuromuscular junction physiology is limited by how closely the fish nAChR resembles the mammalian neuromuscular junction nAChR (i.e., the electric organ is a highly specialized non-muscular organ but of muscle origin and therefore is a neuromuscular junction). However the application of biochemical techniques developed in the electrophysiology studies have only very recently

enabled purification and characterization of the relatively small amount of nAChR present in mammalian skeletal muscle.

Since the nAChR is an integral protein of the muscle membrane, its solubilization in aqueous media requires the presence of detergent. Both nonionic (Miledi and Potter, 1971; Berg et al., 1972; Alper et al., 1974) and ionic (Chiu et al., 1973) detergents have been employed to solubilize free receptors and nAChR-BTX complexes. Extraction of minced (Dolly and Barnard, 1977; Brockes and Hall, 1978) or homogenized muscle membranes (Froehner et al., 1977) in buffered 1-2% triton X-100, the most commonly used detergent, results in a 10-15 fold purification of nAChR with greater than 80% yield.

Solubilized nAChR is commonly assayed by incubation with radioactively labelled  $\alpha$ -BTX (under conditions which minimize proteolysis) followed by separation of free from complexed toxin. Various separation methods based upon the differences in the biochemical behavior of free toxin versus receptor-toxin complex have been utilized including: gel filtration (Dolly and Barnard, 1977; Almon et al., 1974), ion exchange chromatography on carboxymethyl sephadex, differential ammonium sulfate precipitation (Franklin and Potter, 1972), DEAE cellulose disc filtration (Dolly and Barnard, 1977), small scale hydroxylapatite and sepharose bound Concanavalin A chromatography (Mittag et al., 1978).

Attempted purification of muscle nAChR has most commonly utilized denervated muscle owing to the 5-20 fold enrichment in receptors 5-30 days post-denervation. Because of the very low concentration of nAChR in innervated muscle (i.e., 1-4 pmoles/gram wet weight) the most successful purifications have been obtained using two separate affinity chromatography steps and receptors from denervated muscle. The

combination of  $\alpha$ -neurotoxin-sepharose with Concanavalin A-sepharose chromatography -- the latter taking advantage of the glycoprotein nature of the receptor -- has resulted in the purification of denervated rat nAChR to a specific activity of 7.7-10.9 micromoles of  $\alpha$ -BTX binding/gram protein (Froehner et al., 1977). These values are comparable to the highest specific activities obtained for electric fish nAChR (Heidmann and Changeux, 1978). Similar enrichments of receptor have been obtained using either the combination of lectin and  $\alpha$ -toxin (Shorr et al., 1978) or lectin and quaternary ammonium group affinity chromatography (Dolly and Barnard, 1977) for denervated rat muscle receptor. The advantage to using the quaternary ammonium group affinity column and elution of the receptor with the nicotinic antagonist flaxedil is that it avoids conditions that may produce desensitization or agonist induced changes in ligand binding which can occur with the neurotoxin affinity column method (Colquhoun and Rang, 1976). Receptor from the non-fusing muscle culture cell line BC<sub>3</sub>H1 has been purified to a specific activity of 2.6 micromoles  $\alpha$ -BTX binding/gram protein (Boulter and Patrick, 1977) and recently an innervated rat receptor purification to a specific activity of 6.4 micromoles/gram protein has been reported (Kemp et al., 1980).

Preparations of purified cat denervated muscle nAChR (10 micromoles/ gram of protein) exhibit a single band on polyacrylamide gel electrophoresis under non-denaturing conditions (Dolly and Barnard, 1977). Sucrose density gradient centrifugation and gel filtration on sepharose CL 6B gave one major peak of toxin binding activity for free receptor as well as a single peak for toxin-receptor complex. The same preparation subjected to isoelectric focusing yielded one major protein

band having a pI of 5.0. Elution and assay for nAChR on this gel showed a single peak in activity corresponding to the pI 5.0 protein. In common with observations on electrophorus nAChR (Biesecker, 1973) and partly purified rat denervated nAChR (Brookes and Hall, 1975) complex formation between receptor and  $\alpha$ -BTX causes a shift from pI 5.0 to pI 5.3. Brookes and Hall (1975) have reported that receptor-toxin complex prepared from purified junctional nAChR has a pI of 5.15, which is 0.15 units lower than that measured for extrajunctional nAChR, and suggests biochemical differences between normal (junctional) nAChR and the receptor synthesized by the muscle in response to denervation (extrajunctional).

The commonly held assumption that the highest specific activity preparation is the purest molecular form of the receptor is questionable in light of the observation that extensive proteolytic destruction of receptor can occur and protein fragments can be removed without loss of toxin binding (Kemp et al., 1980). Such proteolysis would cause an increase in the measured specific activity but at the same time represent partially degraded nAChR. The recovery of receptor following purification schemes which include an  $\alpha$ -toxin affinity step is 20% at best (Dolly, 1980) and often the yield is substantially lower (Dolly and Barnard, 1977; Froehner et al., 1977; Kemp et al., 1980). Furthermore, the possibility arises that if the original muscle nAChR population was non-homogeneous some selection among receptor subtypes may have resulted from affinity based purification methodologies.

Degradation studies using proteolytic enzymes followed by paper chromatography or amino acid analysis of purified rat and cat denervated nAChR has confirmed the protein nature of the receptor (Froehner et al.,

1977; Dolly and Barnard, 1977). The nAChR from muscle is a glycoprotein as is indicated by very high affinity binding to several sugar selective lectins such as Concanavalin A and lens culinaris; lectins selective for  $\alpha$ -D-mannose and  $\alpha$ -D-glucose moieties (Almon and Appel, 1976; Shorr et al., 1978; Mittag et al., 1978). However, solubilized denervated muscle receptor did not show significant binding to agarose bound lectins which have selectivity for N-acetyl-D-glucosamine (wheat germ agglutinin), D-fucose (fucose binding protein), N-acetyl galactosamine or D-galactose (soybean agglutinin) (Boulter and Patrick, 1977). By comparison with soluble receptor preparations, intact muscle or membrane fragments are not readily labelled with Con A (Dolly, 1979). Consistent with this observation, Con A treatment was shown to be without effect on response of BC<sub>3</sub>H1 cells to iontophoretically applied ACh. However, Con A does block toxin binding to receptor solubilized from these cells (Boulter and Patrick, 1977). These findings suggest that carbohydrate binding sites on the membrane bound receptor may be at least partially hidden from the exterior of the cell membrane.

Gel filtration of  $\alpha$ -BTX-nAChR complex has suggested an apparent molecular weight of 370,000 and 390,000 for cat and rabbit muscle, respectively (Dolly and Barnard, 1977; Brauley et al., 1976). These values are likely to be overestimates resulting from receptor bound detergent. Electropex nAChR has been reported to contain up to 50% bound detergent molecules by weight (Edelstein et al., 1975). Detergent binding gives rise to an increase in molecular weight and buoyancy resulting in erroneous molecular weight determinations for nAChR by gel filtration and other commonly used methods. Sucrose density analysis, which suffers from similar problems, has given an apparent Svedberg

sedimentation coefficient of  $9-9.5 \times 10^{-11}$  for muscle receptor (Froehner et al., 1977; Brockes and Hall, 1975; Devreotes and Fambrough, 1975; Boulter and Patrick, 1977), identical with that observed for electroplax nAChR (Meunier et al., 1974; Lindstrom and Patrick, 1977). This suggests that nAChR from both sources has a very similar if not identical molecular weight. More reliable methods of molecular weight determination using sodium dodecyl sulfate (SDS) disc gel electrophoresis following chemical fixation with glutaraldehyde or suberimide and membrane osmometry have resulted in values of 260,000 (Biesecker, 1973), 275,000 (Hucho and Changeux, 1978), and 270,000 (Martinez-Carrion et al., 1975) for electroplax receptor. By analogy, these findings suggest a molecular weight for muscle nAChR of approximately 270,000 daltons. Larger molecular weight species have been observed in muscle preparations as minor components exhibiting a peak at 13S and greater on sucrose gradient sedimentation (Dolly and Barnard, 1977; Dolly, 1979). These larger species are thought to be aggregates of the 9s nAChR.

There has been some controversy regarding the subunit composition of muscle nAChR. SDS gel electrophoresis of receptor purified from denervated and innervated rat muscle has led to reports from one laboratory of a subunit composition containing 45,000 and 49,000 molecular wt. species as major components as well as minor bands at 51,000, 56,000, and 62,000 daltons (Froehner et al., 1977a; Nathanson and Hall, 1979; Froehner et al., 1977b). Of these subunits, both the 45,000 and 49,000 species have been found to bind the alkylating affinity ligand 4-(N-maleimido)-benzyl-[ $^3\text{H}$ ] trimethylammonium (MBTA) believed to be specific for the ACh binding site (Froehner et al., 1977a). Electroplax nAChR,

on the other hand, exhibits four subunits of approximate molecular weights 40,000, 50,000, 60,000 and 65,000 of which only the smallest binds MBTA (Raftery et al., 1976; Karlin et al., 1976; Rafto and Froehner, 1979). In disagreement with the above, two laboratories studying the composition of highly purified muscle nAChR have reported a single 41,000 or 43,000 m.w. component (or at most two subunits present) suggesting that larger components may be impurities (Shorr et al., 1978; Merlie et al., 1978). Very recent findings indicate that, on the contrary, the findings of a single subunit may be artifactual resulting from a selective purification of the (an)  $\alpha$ -toxin binding subunit which is formed by proteolytic degradation of the receptor complex during the purification procedure (Nathanson and Hall, 1980). Photoactivation in situ of a photoaffinity label derivative of [ $^{125}$ I]  $\alpha$ -BTX which was incubated with muscle membranes labels all five receptor subunits previously reported (Nathanson and Hall, 1980). These results suggest that the  $\alpha$ -toxin binding site on the receptor in situ involves portions of all the polypeptides reported to compose the purified receptor. However, the binding of low molecular weight ligands such as MBTA may be restricted to only two of the polypeptide subunits.

#### D. nAChR-Recognition protein - Pharmacological characterization

The toxin binding characteristics of the nAChR are an important tool for receptor identification. Results of studies on the rate of binding of radiolabelled  $\alpha$ -BTX to solubilized crude (Colquhoun and Rang, 1976) and purified (Dolly and Barnard, 1977) muscle nAChR as well as to muscle membrane preparations (Colquhoun and Rang, 1976; Barnard et al., 1977) have suggested second order behavior for the interaction, i.e. the rate is dependent on both toxin and nAChR concentration. Careful

analyses of the kinetics of  $\alpha$ -BTX binding over several hours (Brookes and Hall, 1975; Mittag et al., 1978; Kemp et al., 1980) and pseudoequilibrium studies over a broad range of toxin concentration (Almon and Appel, 1976) suggest the presence of two classes of sites with different affinities for  $\alpha$ -BTX receptors isolated from both innervated and denervated muscle. Although the reported range of association rate constants is wide (i.e.  $1.5 \times 10^8 \text{ M}^{-1} \text{ min}^{-1}$  (Alper et al., 1974) to  $7.8 \times 10^8 \text{ M}^{-1} \text{ min}^{-1}$  (Mittag et al., 1978)) much of this variability can be attributed to differences in reaction conditions (e.g. temperature) used by various investigators. The fast associating toxin rate constant as measured in solubilized denervated muscle receptor is 7-fold larger than the slower one found by both Mittag et al. (1978) and Kemp et al. (1980) and 4-fold larger than that found by Brookes and Hall (1975). The two binding sites are found to be roughly equivalent in their concentration by two groups (Brookes and Hall, 1975; Mittag et al., 1978). Almon and Appel have reported a much larger affinity difference of approximately 4 orders of magnitude for two sets of  $\alpha$ -BTX binding sites in denervated muscle. This result was obtained from Scatchard analysis, which is applicable only under true equilibrium binding conditions. It is extremely unlikely that equilibrium was attained in their experiments. It is also possible that Almon and Appel are looking at a completely new set of very low affinity toxin binding sites (which are blocked by curare) not seen by others but observed by this group due to the very high [ $\alpha$ -BTX] employed.

Reported measurements of the rate of dissociation of [ $^{125}\text{I}$ ]  $\alpha$ -BTX from soluble receptor are not in agreement with one another. Brookes and Hall (1975) have reported two rate constants for dissociation from

partially purified innervated and denervated rat muscle nAChR which correspond to the two association rate constants they determined. Both innervated and denervated preparations give similar results exhibiting  $T^{1/2}$  values of 3.5 to 4 hrs and approximately 100 hrs for the fast and slow components respectively. Other groups have not observed a biphasic dissociation, the fast dissociating component being absent. Kemp et al. (1980) estimate the  $T^{1/2}$  for  $\alpha$ -BTX dissociation from purified innervated and denervated receptor to be 70-100 hrs, while Colquhoun and Rang (1976) report no evidence of any dissociation within 45 hrs. It is of interest that although the latter group finds no dissociation of  $\alpha$ -BTX from crude muscle extracts, they do observe approximately 30% dissociation from membrane bound receptor after 45 hrs, an observation that suggests solubilization alters the receptor protein. All  $T^{1/2}$  values which are reported in the 45-100 hr. range must be considered as lower limits because receptor proteolysis and denaturation have not been controlled and would contribute to the observed dissociation rate.

It is an open question whether solubilization and purification significantly alters the properties of the membrane bound nAChR. Dissociation constants for  $\alpha$ -BTX calculated from forward and backward rate constants are .04 - .37nM and .02-.17nM for partially purified innervated and denervated rat diaphragm muscle, respectively (Brockes and Hall, 1975). A similar value of .01nM has been obtained for crude membrane homogenates derived from the identical tissue (Colquhoun and Rang, 1976), suggesting no significant solubilization induced alteration. In contrast to the above, Almon and Appel (1976) found by equilibrium measurements that the  $\alpha$ -BTX affinity of a crude extract of receptor from innervated or denervated rat muscle was 10-fold lower than determined by

forward and reverse rate constants. Interestingly, they found an enhanced  $\alpha$ -BTX affinity with similar values to those determined by rate measurements after chromatography of the crude extracts on Con A-sepharose.

Miledi and Potter have studied the binding of  $\alpha$ -BTX to intact frog sartorius muscles by simple immersion of muscle in the toxin solutions (Miledi and Potter, 1971). The number of toxin binding sites per end plate was measured to be  $10^8$ , 60 times that measured for rat muscle. Very high concentrations of dTC ( $10^{-6}$ - $10^{-5}$  M) were able only to inhibit 50% of the total  $\alpha$ -BTX binding, whereas carbachol could inhibit a maximum of 81% of total toxin binding. This curare insensitive binding may represent binding to sites other than the receptor. Porter et al. (1971) also showed that curare could block only 50% of the binding of toxin to the end plate. These authors conclude that there must be two classes of  $\alpha$ -BTX binding sites at the end plate.

Mittag and coworkers (1978) have presented evidence that there are two classes of  $\alpha$ -BTX binding sites in nAChR solubilized from denervated rat muscle. These sites were distinguished by association rate measurements and may reside on different classes of receptors which are physically separable from one another. Receptor heterogeneity was also suggested by the results of another study utilizing sera from patients with Myasthenia Gravis, who have anti-AChR antibodies (Vincent, 1980). Mittag et al. (1976) demonstrated that some patients possess antibodies which prevent approximately 50% of solubilized denervated rat receptor-BTX complex from binding to Con A sepharose affinity gels. Analysis of the association rate of toxin to the 50% of the receptor which does bind to Con A-sepharose after incubation with such sera, suggests this

component to have different toxin binding kinetics from the mixture (Mittag et al., 1978). This has been interpreted to indicate that either the two kinetic components reside on distinct protein molecules, or a single protein which is composed of both components may be separated into its kinetic subunits by antibody directed to the Con A binding site.

Since  $\alpha$ -BTX binds irreversibly to the nAChR, at least on the time scales employed experimentally, the equilibrium binding of small cholinergic ligands has been inferred from measurement of the ligands ability to depress the rate of  $\alpha$ -BTX binding (Colquhoun and Rang, 1976; Barnard et al., 1977; Alper et al., 1974; Fulpius et al., 1973). Under toxin labelling conditions in which  $10^{-3}$ - $10^{-6}$  M ACh, decamethonium or dTC protected 95-100% of the toxin binding sites in purified membrane bound cat nAChR, high concentrations of atropine or pilocarpine showed no measurable depression in toxin binding rate (Barnard et al., 1977). Pharmacologic potency for a series of nicotinic ligands as determined by their antagonism of the toxin binding reaction was found to be consistent with their ability to alter neuromuscular function. For example, similar ligand affinities have been obtained for membrane bound or solubilized nAChR from cat (Barnard et al., 1977) or rat (Colquhoun and Rang, 1976) muscle. Inhibition of toxin binding by agonists or antagonists has been described to be consistent with a simple competitive mechanism as judged by the linear relationship between degree of inhibition and  $\log$  [inhibitor] (Colquhoun and Rang, 1976; Barnard et al., 1977). These findings are in contrast with the more complex type of inhibition (a hyperbolic relation) obtained for receptor preparations from electric tissues (Fulpius et al., 1973). Colquhoun

and Rang have noted that the inhibition of toxin binding by agonists, in contrast to antagonists, developed much more slowly than can be accounted for by the rate of association of agonist with the receptor. This suggests that an agonist induced conformational change in the receptor may be the mechanism for agonist inhibition of toxin binding and that results indicating an apparently competitive interaction may be misleading since this may not result from direct competition for the same binding site.

More direct evidence that the interaction of nicotinic agonists and antagonists with the  $\alpha$ -BTX binding site on the nAChR may not be a simple competition has been presented by Eldefrawi and coworkers (Sarvey et al., 1978). Low concentrations of carbachol and decamethonium as well as dTC offer protection from irreversible inhibition by  $\alpha$ -BTX of muscle tension in the indirectly stimulated rat diaphragm muscle. Although  $\alpha$ -BTX does not irreversibly inhibit the muscle response in the presence of these agonists and antagonists, as occurs in their absence, a completely reversible inhibition is obtained in their presence. Since the agonists offer complete protection from the irreversible action of  $\alpha$ -BTX, they must occupy the receptor under these conditions. However, since  $\alpha$ -BTX completely inhibits the response, even though the protecting ligands are present,  $\alpha$ -BTX must also be bound to the receptor. This has been explained by the proposal of two-step kinetics for the binding of  $\alpha$ -BTX to the receptor similar to that already suggested by several groups in studies of purified electroplax receptor (Bulger and Hess, 1973; Klett et al., 1973; Raftery et al., 1972). It is proposed that reversible binding of  $\alpha$ -BTX precedes the formation of an irreversible complex, the latter may be blocked by agonists and possibly antagonists (Sarvey et

al., 1978).

#### E. nAChR Recognition Protein-Chemical Modification

nAChR contains a reducible sulfhydryl group in the vicinity of the ACh binding site on the receptor which alters receptor function. Dithiothreitol (DTT), an effective reducing agent of disulfide bonds in proteins, decreases the apparent affinity of the receptor for many nicotinic agonists. In electroplax (Karlin, 1969; Karlin, et al., 1966), frog rectus and sartorius (Mittag, et al., 1970; Fleisch, et al., 1979), chick biventer cervicus (Rang, et al., 1971), denervated rat (Albuquerque, et al., 1968), and leech muscle (Ross, et al., 1972) the log dose-response curves to acetylcholine or carbachol are shifted 3-30 fold to the right after treatment with DTT. The reduction in responsiveness to these agonists is fully reversed by treatment with oxidizing agents such as ferricyanide or 5,5'-dithio-bis (2-nitrobenzoate) (DTNB). In contrast to the decrease in apparent affinity for carbachol and ACh induced by DTT treatment, a 2.4 fold increase in apparent affinity for curare has been observed (Rang, et al., 1971). These observations cannot be attributed to a simple inactivation of a fraction of the receptor population because unlike the other pure agonists the partial agonist properties of decamethonium change so that it becomes a more potent agonist after DTT treatment (Karlin, 1969; Rang, et al., 1971). In DTT reduced tissue there have been reports of qualitative (intrinsic activity) as well as quantitative (potency) differences in response to nicotinic cholinergic drugs. Hexamethonium, which behaves as an antagonist in naive muscle and electroplax tissue, becomes an agonist following treatment with DTT in electroplax and chick muscle (Rang, et al., 1971; Karlin, et al., 1968) although this has been

found not to be the case for frog (Ben-Haim, et al., 1973) or leech muscle (Ross, et al., 1972). Changes in the intrinsic activity of ligands following receptor modification with DTT suggest that a specific disulfide bond is reduced in the receptor complex to form a modified receptor complex with unique ligand-binding properties.

A detailed study of the responsiveness of isolated strips of chick muscle to a series of mono- and bis- quaternary cholinergic drugs, following reduction with DTT, has been carried out by Rang and Ritter (1971). After reduction, the muscle became less sensitive to monoquaternary agonists, however, bisquaternaries of the decamethonium series with chain lengths  $C_6$ - $C_9$  became more potent agonists. A plot of agonist potency versus chain length for the series suggests that agonist activity after DTT treatment corresponds to the activity of the homolog with the carbon chain longer by one methylene group than on the unreduced preparation. This finding suggests that there is a small increase in the distance between adjacent cationic binding sites on the receptor, i.e., reduction changes the configuration of the ligand binding site(s) (Rang, et al., 1971).

Additional evidence that the observed alteration in pharmacological potency induced by DTT is due to an action at the nAChR comes from attempts at preparing site directed drug molecules. Although reoxidation of receptors occurs with DTNB or ferricyanide, relatively high concentrations are needed for reversal of DTT effects in muscle bath experiments (approx. millimolar). The incorporation of a quaternary ammonium group into an oxidizing agent has been found to greatly enhance its ability to reoxidize receptors (Bartels, et al., 1970). Bartels has shown that dithio-bis-choline, which consists of two

molecules of thiocholine coupled through a disulfide bond, is approximately 5,000 times more potent at oxidizing reduced receptors than is DTNB (Bartels, et al., 1970).

Alkylation of reduced nAChR in electropex with N-ethyl-maleimide (NEM), at a concentration without an effect on unreduced receptors, has been shown to eliminate the ability of DTNB to reoxidize receptors (Karlin, et al., 1966). Irreversible inhibition of responsiveness to ACh or carbachol results from NEM treatment of reduced but not untreated electropex tissue (Karlin, et al., 1966). In an attempt to confer specificity for alkylation on the reduced receptor Karlin and coworkers prepared alkylating maleimide derivatives which contain moieties having known nicotinic cholinergic action (Karlin, et al., 1968; Karlin, 1969) i.e., site-directed alkylating agents. Testing of 4-(N-maleimido) phenyltrimethylammonium iodide (MPTA) and 4-(N-maleimido) benzyltrimethylammonium iodide (MBTA) revealed a 2,500-fold and a 4,650-fold increased rate of block of the maximal response to carbachol relative to that observed with NEM (Karlin, 1969). Karlin (1969) showed that only a fraction of the enhanced activity of MPTA and MBTA, 5.5- and 4.3-fold, respectively, could be attributed to increased reactivity with cysteine residues over that determined for NEM. He therefore concluded from the observed site directedness induced by the phenyltrimethylammonium group that the reducible disulfide bond (whose reduction is responsible for altered pharmacological potency of nicotinic ligands) is in the immediate vicinity of the cationic binding site on the receptor for acetylcholine. Further support for this site of action is provided by the observation that hexamethonium, a selective antagonist, protected the reduced receptor against irreversible inactivation by MPTA (Karlin, et

al., 1968). Thus, in electropex, MPTA and MBTA exhibit the critical properties expected of an affinity labelling reagent, affinity enhancement of reactivity, and, by the criterion of protection experiments, specific labelling of the active site. Confirmation of this selectivity has come from isolation of labelled receptor subunits following irreversible labelling with radioactive alkylators.

Studies on the effects of alkylating agents following DTT reduction in frog muscle are not all in agreement with results on electropex (Karlin, 1969) that the reducible disulfide bond is in the immediate vicinity of the ACh binding site. Lindstrom et al. (1973) found that although MPTA and MBTA irreversibly reduced the maximum carbachol response after DTT reduction of frog sartorius muscle, these agents were no more effective than NEM. Neither carbachol ( $25\mu\text{M}$ ) nor dTC ( $10^{-5}\text{M}$ ) offered any measurable protection against inactivation produced by MPTA alkylation. These results were interpreted to indicate that there is no reducible disulfide bond within 10-20 Å of the ACh binding site in frog muscle. However, a more likely explanation (in the light of other evidence) is that the optimal distance between the quaternary ammonium group and the reactive site in the alkylating ligand is not 12 Å as inferred from experiments with electropex (Karlin, 1969). These data suggest that different nAChRs may have slightly different configurations at the drug (ligand) binding site. In contrast to the findings of Lindstrom et al. (1973) results have been reported which suggest that the reactive disulfide group in frog muscle responsible for altered pharmacology upon DTT reduction, does reside in the immediate vicinity of the ACh binding site on the receptor (Ben-Haim, et al., 1973). Bromoacetylcholine (BACA), an active site-directed reagent, was found

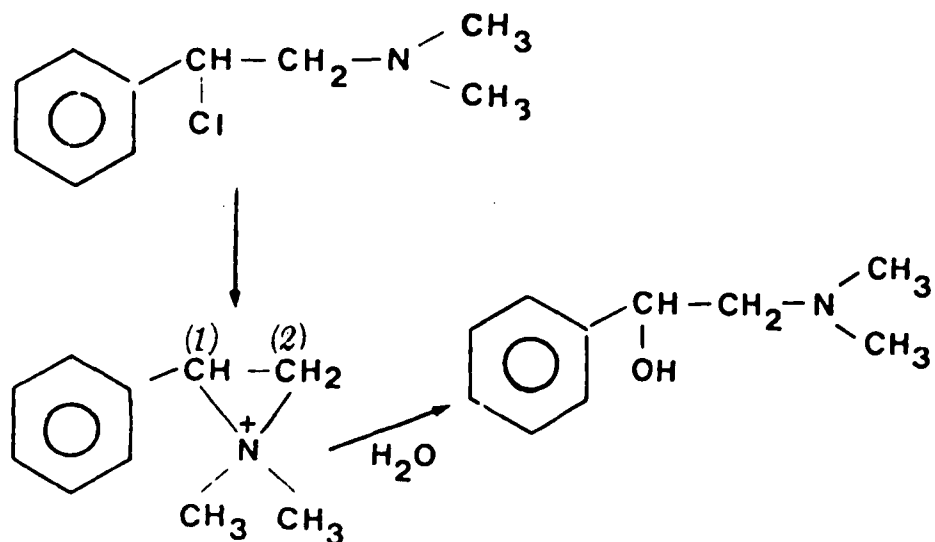
in micromolar concentrations to prevent reoxidation of the receptor by DTNB. The selectivity of BACA is suggested by the observation that reoxidation was not prevented by millimolar concentrations of bromoacetic acid which lacks the quaternary ammonium group of BACA. The effect of low concentrations of BACA was prevented by hexamethonium which presumably protects against alkylation by occupying the ACh binding site on the receptor. The authors interpret these findings to indicate that the "reactive" disulfide group lies within a few angstroms of the ACh binding site.

In addition to alkylating agents which behave as irreversible nicotinic cholinergic antagonists, irreversible agonist activity has been reported. Treatment of reduced electroplax tissue with bromoacetylcholine or (p-carboxyphenyl) trimethylammonium iodide results in a sustained depolarization that is not reversed by washing (Silman, et al., 1969). The "irreversible" agonist activity observed with these compounds, presumably due to covalent attachment at the ACh binding site, is blocked by adding dTC. Washout of the dTC causes a reappearance of previously measured depolarization. These findings have been interpreted to indicate that the quaternary group is free to engage and disengage with the receptor while remaining fixed to the sulfhydryl group which is in the vicinity of but not actually in the active site (Silman, I. et al., 1969).

Similarly, Mittag et al. (1970) observed an irreversible contracture in reduced frog rectus abdominis muscle after alkylation with iodoacetylcholine, N,N-dimethylphenylaziridinium (DPA) and acryloylcholine. At least in the case of iodoacetylcholine non-selective actions were ruled out by the observation that a high

concentration of iodoacetic acid ( $2 \times 10^{-4}$  M) had no direct effect either before or after DTT treatment and did not alter responses to ACh either before or after DTT. It is unlikely that an irreversible agonist response obtained in reduced muscle with an alkylating agent that is a simple reversible cholinergic agonist when acting on untreated muscle would be due to a non-selective interaction. These findings are consistent with the notion that the active site for agonists of frog muscle nAChR in common with electroplax tissue has a disulfide bond in close proximity to the cationic binding site for ACh on the receptor. The data also suggest that the antagonist binding site may be separate from the agonist site and also has a disulfide in close proximity, perhaps.

The present study utilizes the alkylating agonist (DPA) to irreversibly modify the nAChR. DPA is an analogue of the reversible cholinergic agonist phenyltrimethylammonium. The DPA ion is rapidly formed, at physiological pH, from its 2-chloroethylamine precursor as follows:



2-Haloalkylamines are known to be effective alkylating agents due to their ability to generate the aziridinium ion. Numerous drug molecule types containing the aziridinium moiety have been found to bind irreversibly to a number of receptors and enzymes. With an appropriate affinity group to direct its site of alkylation it is possible to achieve a fair degree of potency and selectivity. DPA, a quaternary compound, might be expected to interact with the nAChR to form a reversible complex. If a nucleophilic moiety, such as a sulfhydryl group, were in close proximity to the reactive aziridinium ring a stable covalent bond might be found.

DPA has been reported to cause acute flaccid paralysis with skeletal muscle fasciculations in cats along with depressed response to motor nerve stimulation (Hunt, 1949). It blocks indirectly contractions of rat diaphragm but not those elicited by direct stimulation (Mittag, et al., 1970). On a slow muscle, the frog rectus abdominis, DPA behaves as an agonist causing contracture (Mittag, et al., 1970). The properties of the contracture observed on the rectus preparation are what one would expect for a cholinergic agonist. The known effects of DPA on neuromuscular systems are in agreement that it is a depolarizing agent.

### 3. nAChR-Ion conductance channel

#### A. Electrophysiologically deduced characteristics.

The binding of one or more molecules of ACh to the recognition site(s) on the nAChR induces a conformational change in the receptor which results in a transient increase in cation permeability. The ACh induced increase in cation conductance occurs via a specific ion conductance channel (ICC) which may be an integral part of the receptor

protein or possibly a distinct but conformationally linked protein molecule (i.e. a conformation change in the recognition protein gives rise to a conformational alteration in the ICC). The miniature end plate current (MEPC) results from the release of one quanta of ACh, estimated to be 10,000 molecules (Hartzell et al., 1976), into the synapse and is the summation of approx. 2,000 individual elementary channel opening events. The end plate current (EPC) is in turn composed of some multiple of MEPCs.

The brief increase in conductance which results from ACh action gives rise to a transient membrane depolarization (End plate potential, EPP) to a value of -15 mv in frog motor end plate (Fatt and Katz, 1951). This value of -15 mv has been determined to be the reversal potential, i.e. the membrane potential at which no net current flows in response to the action of transmitter (Del Castillo and Katz, 1954). Since the reversal potential does not coincide with the equilibrium potential for  $\text{Na}^+$ ,  $\text{K}^+$  or  $\text{Cl}^-$ , the only ions present in high concentration, it was apparent that the conductance increase resulting from ACh action must involve more than one ion. Altering the external concentration of an ion would be expected to change its equilibrium potential. If the ion in question contributes to the endplate current, the reversal potential would be expected to shift to a new value in response to its altered equilibrium potential. Using this criterion, Takeuchi and Takeuchi (1959) showed that it is an increased conductance to sodium and potassium, but not chloride, which is responsible for the end plate current produced by ACh. The ratio of sodium to potassium conductance through the ICC has been calculated to be 1.3; the ions being approximately equally well conducted. Although only a very small

fraction of the current is normally carried by divalent cations, their permeability has been found to increase by ACh induced channel opening. Depolarization of the end plate has been measured under conditions where calcium was used to completely replace sodium in the bathing solution (Takeuchi, 1963). Katz and Miledi (1970) demonstrated that miniature end plate potentials could be recorded in frog muscle bathed in isotonic calcium or magnesium chloride (containing 2mM  $K^+$ ). In addition to conductance of inorganic cations, the ion channel associated with the nAChR has been found to conduct organic cations. Creese and coworkers (Creese and MacLagen, 1971; Creese, 1971) have shown by autoradiography that labelled decamethonium, a depolarizing antagonist that can trigger its own entry, enters rat diaphragm muscle fibers and that permeation is blocked by low concentrations of dTC. It has been proposed that entrance is through ion channels associated with the nAChR in the end plate, which decamethonium opens. Methylamine, dimethylamine, ethylamine and isopropylamine have been demonstrated by Furokawa and Furokawa (1959) to be capable of substituting for sodium while retaining the ability of the endplate to depolarize in response to transmitter. There is also evidence that acetylcholine enhances permeability to hydrazine (Koketsu and Nishi, 1959), ammonium and tetramethylammonium ions (Nastuk, 1959).

Utilization of the voltage clamp technique has enabled measurement of the time course of the EPC and MEPC. The rise time of these ACh induced ion currents is very short. The amplitude of the endplate current reaches a peak in 0.5-1 msec (Takeuchi and Takeuchi, 1959; Magleby and Stevens, 1972; Kordas, 1969) while the miniature end plate current peaks in less than 300  $\mu$ sec (Gage and Armstrong, 1968; Gage and

McBurney, 1971). The more prolonged time for the EPC to reach its peak amplitude as compared with the MEPC is thought to be due to non-synchronous release of quanta (Barrett and Stevens, 1972) and imperfect summation of MEPPs in time. Thus, it appears that rate of growth of the EPC is limited by the rate of change of ACh concentration in the micro-environment of the recognition site on the receptor. After reaching a peak, both the EPC (Takeuchi and Takeuchi, 1959; Magleby and Stevens, 1972) and the MEPC (Gage and McBurney, 1971) decline at an exponential rate with time constants of 1-3 msec. In frog sartorius muscle at 17°C, the exponential decay of the end plate current had a half time of 1.1 milliseconds, in good agreement with the time course of the end plate potential (Takeuchi and Takeuchi, 1959). The fact that the decay rate of these ACh dependent currents is exponential suggests that a first order reaction controls the rate of decay of the conductance. This limiting first order process may be the dissociation of ACh from the recognition site that is followed by a more rapid reversion of the channels to the "closed" non-conducting form of the ICC. Alternatively the rate limiting step may be the conformational shift from "open" to "closed" channels and may be independent of acetylcholine dissociation rate. In support of the latter possibility, Stevens et al. (1975) have presented evidence that ACh dissociates from the receptors and is completely hydrolyzed by acetylcholinesterase within only a few hundred  $\mu$ sec. indicating that ACh dissociation cannot be rate limiting.

The time constant for the decay of the endplate is temperature and voltage dependent. Q10's for the temperature dependence have been reported with a value of 2.5 (Takeuchi and Takeuchi, 1959; Kordas, 1972) in agreement with a Q10 of 3 for the mean life time of the elementary

conductance event induced by ACh (Anderson and Stevens, 1973). The decay of end plate currents is progressively shortened as the membrane is depolarized (Takeuchi and Takeuchi, 1959; Kordas, 1969; Magleby and Stevens, 1972). Both the rate of opening and the rate of closing of the channel increases, approximately doubling, for each 100 mv depolarization of the membrane. Although early voltage clamp experiments indicated a linear relationship between amplitude of the EPC and clamped potential (Takeuchi and Takeuchi, 1959), a small deviation from linearity has been noted (Magleby and Stevens, 1972; Kordas, 1969). It has been proposed that the observed deviation (which indicates that the membrane potential may not simply be the driving force for the EPC) may stem from an effect of membrane potential on the stability of the ACh-receptor complex (Kordas, 1969).

It has become possible through the use of sophisticated recording techniques to measure discrete individual conductance events at the endplate which are believed to be the opening and closing of single ICCs. These statistically random conductance events have a constant amplitude (under a given set of conditions) and a square pulse shape (Neher and Sakman, 1976; Neher and Steinbach, 1978). Statistical analysis of the "noise" results from current fluctuations enables the evaluation of the channel relaxation time and mean channel conductance (Neher and Stevens, 1977). Typical values of 1-3 msec and 15-30 pOhm have been obtained, respectively. Katz and Miledi (1972) have obtained a value of .25 $\mu$ V for the elementary potential from which they calculate that each channel must conduct  $10^{-14}$  coulombs of charge or 60,000 univalent cations. Disulfide bond reduction with DTT has been found to reduce the single channel conductance by about 40% by shortening of the channel

life time (Ben-Haim et al., 1975). The mean channel life time and the amplitude of the single channel conductance were studied for a variety of cholinergic agonists (Katz and Miledi, 1973). It was observed that the amplitude of the conductance was constant for each of the simple agonists studied, however there was wide variation in the observed channel life time. Results for the mean channel life time spanned a 10-fold range, from 1.65 msec for suberyldicholine to .12 msec for acetylthiocholine. These findings suggest a possible physical basis for drug efficacy.

A comparison of the characteristics of the ACh-sensitive endplate channel with those of the voltage-sensitive sodium channel found throughout the muscle membrane, indicates a clear distinction between the two different channels. Channels can be distinguished from one another on the basis of the rate constants for channel opening and closing, channel density, and pharmacological specificity of agents which block or alter the kinetics of the conductance event (Rang, 1975). Pharmacological agents have not been found which differentially effect the conductance of sodium and potassium through the ACh-sensitive endplate channel (receptor coupled) suggesting that unlike the voltage sensitive cation channels, a single endplate channel likely conducts both ions.

#### B. Pharmacological Modifiers of the Ion Conductance Channel.

Biophysical analysis of the acetylcholine conductance channels in the presence of pharmacological agents has suggested that drugs can act directly on the ion conductance channel at a discrete site(s) and may be distinguished from those that act at the ACh recognition site. Agents such as  $\alpha$ -BTX (Changeux et al., 1970; Katz and Miledi, 1973) and

nereistoxin (Eldefrawi et al., 1980) are thought to act purely at the recognition site to diminish the amplitude of the end plate current without effect on its time course; these drugs act in a voltage independent manner. By contrast drugs which interact with the conductance channel diminish the amplitude of the endplate current and alter its time course and do so in a voltage-dependent manner. The voltage-dependence of the blockade by drugs of this class is characterized by a deviation from the normal approximate linearity of the endplate current-voltage relationship. Antagonists at the neuromuscular junction which have been reported to act on the ion conductance channel include the amine containing local anesthetics (Steinbach, 1978; Adams, 1975; Gage and Armstrong, 1968), histrionicotoxin (HTX) and derivatives (Albuquerque et al., 1974; Eldefrawi et al., 1977; Albuquerque et al., 1973), amantidine (Tsai et al., 1978), scopolomine (Adler et al., 1978) and phencyclidine (Tsai et al., 1979; Albuquerque et al., 1980). Other agents such as quinacrine (Tsai et al., 1979) and tetraethylammonium (Adler et al., 1979) have been reported to interact with ICCs, but also block the ACh recognition site in the same concentration range. These agents can block ACh-induced depolarization by a dual mode of action. dTC, which is usually considered to be the prototype receptor blocking agent, also blocks ICCs at one order of magnitude higher concentration (Tsai et al., 1978).

Historically, local anesthetics were the first drugs which were investigated as channel blockers at the neuromuscular junction. In the presence of local anesthetics, such as procaine (Adams, 1975) and lidocaine derivatives (Steinbach, 1968; Ruff, 1977), the decay of the ACh induced endplate current does not fit a single exponential time

course. The multicomponent decay time courses observed with local anesthetics have been resolved into the sum of at least two exponential components; an initial component which decays more rapidly than that observed in the absence of local anesthetics as well as a prolonged decay component which is slower than that of the control. In addition to these faster and slower than normal EPC decay rates, a third exponential decay has been observed in the presence of Qx222, a lidocaine derivative. The third component has a time course similar to the control EPC and is thought to represent unmodified receptors (Beame, 1976; Ruff, 1977).

Changes in the decay of the EPC by local anesthetics were accounted for by Ruff (1977) by a three state model of the agonist bound receptor, i.e., non-conducting, conducting, and blocked; each state is capable of binding drugs. Increasing the local anesthetic concentration or hyperpolarizing the membrane increases the rate at which the conducting state shifts to the blocked state. Consistent with this model it was found that single channel currents in the presence of lidocaine derivatives (Qx222 and Qx324) did not decay as the sum of two exponentials (Neher and Steinbach, 1978). The square pulse wave forms believed to represent the opening and closing of ACh induced elementary conductance channel are chopped into a series of much shorter duration events in the presence of the lidocaine derivatives. The flickering current is believed to be the local anesthetic repeatedly and reversibly interacting with and blocking the open ICCs. Rates for single channel closing, blocking and unblocking were measured and found to be voltage dependent. The flickering rate (blocking) was linearly related to local anesthetic concentration while the rate of unblocking was found to be

concentration independent. It was concluded from these studies (Neher and Steinbach, 1978) as well as others (Horn et al., 1980; Adams, 1977) that local anesthetics enter ACh induced open ICCs and transiently occlude them by binding to a site on the channel approximately three quarters of the way through the membrane. Utilizing internal perfusion techniques to administer quaternary lidocaine derivatives, it has been found that high intracellular concentrations of local anesthetic (1 mM) are ineffectual at blocking the nAChR linked ICC (Horn et al., 1980). Since these local anesthetic concentrations are well in excess of that required for channel blockade when applied extracellularly, a basic asymmetry of the channel has been suggested.

Probably the most well studied of the compounds reported to block the nAChR linked ICC, and generally considered to be the prototype for this activity, is HTX, an alkaloid isolated from the skin of the Colombian frog Dendrobates histrionicus (Daly et al., 1971). In common with local anesthetics, HTX and somewhat more potently its reduced derivative perhydro-histrionicotoxin (H<sub>12</sub>-HTX) reversibly reduce the amplitude and alter the time course of the EPC at frog and mouse neuromuscular junction (Albuquerque et al., 1974; Gage et al., 1975; Masukawa and Albuquerque, 1978; Lapa et al., 1975). HTX and H<sub>12</sub>-HTX shorten the falling phase of the EPC as has also been observed for atropine (Adler et al., 1978). This behavior on EPC time course is in contrast to that previously mentioned for local anesthetics which shows an initial shortening followed by a prolongation of the ACh conductance. The significance of this difference in the effects of local anesthetics and HTX is presently unclear.

The degree of inhibition of the EPC by a fixed concentration of HTX

is voltage-dependent, HTX being a more potent inhibitor as the membrane is progressively hyperpolarized (Albuquerque et al., 1974; Masukawa and Albuquerque, 1978). Thus, there is a marked deviation from linearity in the relationship between EPC and membrane polarization. The basis for this deviation from the normal linear current-voltage relationship is an increased blockade of closed channels by HTX as the membrane is hyperpolarized (Masukawa and Albuquerque, 1978). This results in a smaller population of channels which may be opened by agonist. Furthermore, the deviation from linearity is not observed when short duration (10 ms) as opposed to long duration (3 s) membrane voltage conditioning steps are applied before measuring the EPC, indicating a time dependence for the additional binding of HTX to closed channels.

It is a reasonable assumption that an agent which interacts with the ICC will have different selectivities and/or affinities for particular conformational states of the nAChR-channel complex. Inhibitory action of HTX on the EPC has also been found to be stimulation dependent, that is, channel function is affected by the preceding channel activity. Various states of the channel have a lifetime longer than the stimulus which causes their formation (i.e. conformational memory even if the inducing ligand has dissociated). Repetitive stimulation of the nerve in innervated muscle or repetitive microiontophoretic application of ACh in denervated muscle causes a progressive decline in the endplate potential in the presence of HTX (Lapa et al., 1975). A similar decline is not observed in the presence of dTC which under the experimental conditions is believed to block the receptors only (Eccles et al., 1941). If channels are allowed to "recover" by not being stimulated following a prior series of stimuli, then the first EPP of the new train

is of the same magnitude as the first EPP of the previous train (Lapa et al., 1975). These findings suggest that HTX may possess higher affinity for the ACh activated-open form of the conductance channel.

Alternatively, it has been proposed that HTX may have its maximal affinity for the desensitized conformation of the receptor-channel complex.

This explanation was based on the apparent enhancement of agonist induced desensitization by  $H_{12}$ -HTX in cultured chick muscle cells (Burgermeister et al., 1977).  $H_{12}$ -HTX has been found to non-competitively inhibit nAChR-dependent  $^{22}Na^{+}$  uptake in this system. The concentration of carbachol required for desensitization as assessed by tissue responsiveness or indirectly by ligand binding is greatly reduced in the presence of  $H_{12}$ -HTX. Consistent with the well known dependence of agonist induced desensitization on  $Ca^{++}$  (Manthey et al., 1966), the  $H_{12}$ -HTX enhancement of desensitization was found to have a similar  $Ca^{++}$  dependency. In summary, HTX has been suggested to interact with all three proposed conformations of the receptor-channel complex. Its action cannot be attributed to a specific blockade of a particular state of the ICC.

Verification that HTX and its derivatives do not interact with the nAChR at concentrations which antagonize the ACh response has come from radioligand binding studies. The retardation by  $H_{12}$ -HTX of the rate of  $\alpha$ -BTX binding to denervated skeletal muscle membranes and solubilized nAChR has been studied (Dolly et al., 1977). While half maximal neuromuscular blockade in junctional and extrajunctional rat muscle was found at  $8.4\mu M$  and  $2.4\mu M$   $H_{12}$ -HTX, respectively, half maximal effect on the kinetics of  $\alpha$ -BTX binding required 10-fold greater concentrations. Furthermore, the action of  $H_{12}$ -HTX at these high concentrations was pure non-competitive with  $\alpha$ -BTX suggesting an allosteric action. Effects of channel

blockers on receptor site ligands would be observed if the channel is an integral component of the receptor protein complex or if the two form a macromolecular complex. Similar high concentrations of  $H_{1,2}$ -HTX were found necessary to block [ $^3H$ ]-ACh binding to electroplax membranes (Kato and Changeux, 1976; Eldefrawi and Eldefrawi, 1975).

The binding of [ $^3H$ ]- $H_{1,2}$ -HTX to electroplax membranes has revealed a single saturable population of sites which have a  $K_d$  of .1-.4 $\mu$ M (Eldefrawi and Eldefrawi, 1975; Eldefrawi et al., 1977; Elliot and Raftery, 1977; Eldefrawi et al., 1978). nAChR antagonists such as  $\alpha$ -BTX, dTC and najatoxin did not compete for [ $^3H$ ]- $H_{1,2}$ -HTX binding, however the ligand was displaceable by local anesthetics, HTX derivatives and several other compounds shown electrophysiologically to alter the EPC time course. Cholinergic agonists such as carbachol were reported to inhibit (Eldefrawi et al., 1977), enhance (Elliot and Raftery, 1977; Albuquerque et al., 1980) or not affect (Eldefrawi and Eldefrawi, 1975; Eldefrawi et al., 1978)  $H_{1,2}$ -HTX binding. An excellent correlation coefficient has been obtained (.96) between the physiological potency and ability to displace  $H_{1,2}$ -HTX bound to electroplax membranes for a series of HTX analogues (Eldefrawi and Eldefrawi, 1975). The reported stoichiometry for the ratio of  $\alpha$ -BTX to  $H_{1,2}$ -HTX binding sites has ranged from 4 (Elliot and Raftery, 1977) to .5 (Eldefrawi and Eldefrawi, 1977). Binding of  $H_{1,2}$ -HTX has been found to increase linearly with increasing protein concentration and be inhibited by heat treatment, proteases, exposure to a sulfhydryl reducing agent (p-chloro mercuribenzenesulfonic acid) or a carboxylate reagent (N-ethoxycarbonyl-2-ethoxy-1,2,-dihydro-quinodine); all suggesting the binding site to be proteinaceous in nature (Eldefrawi and Eldefrawi, 1975). Solubilization of the [ $^3H$ ]-

$H_{12}$ -HTX binding protein has been achieved using 0.5% cholate (Eldefrawi et al., 1978) alleviating the previously reported problem of non-specific binding of the radioligand to Triton X-100 (Eldefrawi et al., 1977). Saturable binding with a  $K_d$  of  $0.2\mu M$  has been reported for [ $^3H$ ]- $H_{12}$ -HTX to cholate extracts of electroplax membranes. The specificity of drugs competing for this binding is consistent with a binding site on the ICC.

HTX is not readily available because the current Colombian government has banned the exportation of *Dendrobates histrionicus* skins. New compounds which possess specificity for the ICC have been sought as research tools to fill this gap. Phencyclidine (PCP), a drug which has received notariety as a result of widespread street abuse, may prove to be of value in this respect.

PCP diminishes the amplitude and time course of both the EPC and MEC in frog sartorius muscle (Albuquerque et al., 1980). This action is slowly reversible and voltage dependent,  $10\mu M$  PCP resulting in half maximal inhibition of the peak amplitude of the EPC at a holding potential of 90 mv. Non-linearity of the EPC-voltage relationship has been observed when the steps in membrane potential were held for 3s but not for 25 ms. Thus, in common with HTX, the voltage-dependent action of PCP is time dependent as well. Since the voltage-and time-potentiated effect of PCP action has been demonstrated not to require previous activation of the nAChR, it has been attributed to enhanced interaction of the drug with closed as opposed to open ion channels. Additional evidence that PCP may interact with closed ion conductance channels is suggested by different dose-response relationships for effect on the EPC amplitude as opposed to time course of EPC and on MEPC amplitude as

opposed to the time course of MEPC. If the effect of PCP were the result of interaction with open ion conductance channels alone, reduction in the time course of the EPC would be expected to account quantitatively for the diminution in EPC amplitude; this has been found not to be the case. Therefore, the authors (Albuquerque et al., 1980) conclude that the observed depression of the EPC by PCP is the sum of the individual effects on open and closed channels.

Competition binding experiments for [ $^3\text{H}$ ]- $\text{H}_{12}$ -HTX to sites presumed to be the ion channel in electroplax membranes are consistent with the suggestion that PCP has its site of action in common with HTX (Albuquerque et al., 1980). PCP was found to inhibit [ $^3\text{H}$ ]- $\text{H}_{12}$ -HTX binding with a  $K_i$  of  $6.9\mu\text{M}$ , a concentration similar to that required for its half maximal physiological effect. Analysis of binding curves showed that PCP competitively inhibits [ $^3\text{H}$ ]- $\text{H}_{12}$ -HTX binding, suggesting interactions at a mutually exclusive site. The binding of PCP was found to be enhanced by the presence of the agonist carbachol; half of maximal stimulation by carbachol was achieved with a concentration of  $60\text{nM}$ . Linear Scatchard plots were obtained with  $K_d$  of  $2.0\mu\text{M}$  and  $10.3\mu\text{M}$  for the binding of PCP in the presence or absence of  $10\mu\text{M}$  carbachol, respectively. These findings suggest that although PCP appears to have affinity for both open and closed ICCs, it may have a higher affinity for open channels. Alternatively, channels associated with desensitized receptors might be "different" conformationally and bind PCP with higher affinity.

#### 4. Interrelation of the nAChR and ICC

One fundamental question regarding the relation of the nACh receptor protein to the channel is whether both the functions of

transmitter recognition and of ion translocation reside in the single polypeptide complex. Attempts at resolving this issue have focused on reconstitution of purified nAChR into lipid vesicles and looking for the concomitant appearance of functional properties such as  $\text{Na}^+$  flux in response to cholinergic agonists, receptor desensitization and allosteric effects by channel blocking agents. Kemp et al. (1973) have incorporated partially purified rat innervated and denervated rat nAChR into phospholipid bilayers and found a four-fold increase in membrane conductance to  $\text{Na}^+$  and  $\text{K}^+$  with ACh addition. Although this conductance increase was blocked by prior treatment with  $\alpha$ -BTX or dTC, the conductance increase was very low and the background conductance very high; suggesting some essential component such as the lipid composition may not be fully controlled. Reconstitution of partially purified rabbit nAChR into planar phospholipid bilayers has resulted in increased conductance in the presence of carbachol (Bradley et al., 1976). The carbachol enhanced conductance was inhibited by only 67% in the presence of  $10^{-6}$  M dTC and by 55% by  $8\mu\text{M}$   $\alpha$ -BTX. Furthermore, extremely high concentrations of carbachol ( $2.5 \times 10^{-4}$ ) were necessary to achieve a maximal conductance increase.

It has long been known that membrane fragments from electroplax form closed vesicles which transport  $\text{Na}^+$  ions in response to added agonist (Kasai et al., 1971). However, reports of a successful reincorporation of purified electroplax, nAChR into membranes with significant restoration of agonist induced sodium flux have not been very successful; and when observed, only a partial success has been obtained (Michaelson et al., 1974). These negative findings suggest a lack of some necessary component in reconstitution experiments.

An explanation for the lack of success in reconstitution experiments utilizing purified nAChR has been suggested by Eldefrawi et al. (1977) on the basis of [ $^3$ H]-H<sub>12</sub>-HTX binding experiments. The binding site for this radioligand on triton x-100 solubilized electroplax membranes, presumably the ICC, was found to be separable from the [ $^3$ H]-ACh binding site by affinity chromatography or immunoprecipitation of the receptor. Sephadex G-200 chromatography was also found to separate the ACh binding and the H<sub>12</sub>-HTX binding proteins, suggesting that the ICC is a protein separable and distinct from the nAChR. These studies, it was noted, were complicated by the fact that Triton X-100 also bound H<sub>12</sub>-HTX in a non-specific and non-saturable manner. A report from the laboratory of Changeux has provided support for the conclusion that the HTX- and ACh binding site are present on separate proteins (Sobel et al., 1978). In electroplax membranes binding of the fluorescent local anesthetic quinacrine gives a signal which is enhanced by carbachol and inhibited by other non-fluorescent local anesthetics (Grunhagen et al., 1976). The resultant increase in fluorescent signal is believed to be due to binding of the fluorescent ligand to the ion conductance channel. A quinacrine binding protein having a subunit molecular wt. of 43,000 daltons and reasonably named the "43,000 protein," has been found to be a major component of enriched electroplax membranes (Sobel et al., 1978). The 43,000 protein has been completely separated from the nAChR on a sucrose gradient following detergent solubilization. Binding of quinacrine to this isolated protein is inhibited by HTX. In contrast to the crude membrane preparations, carbachol did not enhance fluorescence.

The suggestion that the 43,000 protein may represent the ion channel, or one of its components, is in sharp contrast to some very recent

reports of reconstitution of a functional nAChR system in the absence of the 43,000 protein. Several laboratories have reported that in enriched electroplax membranes the 43,000 protein may be selectively removed by alkaline extraction without removing from the membranes the capacity to respond as a functional receptor channel complex (Sobel et al., 1980; Heidmann et al., 1980; Moore et al., 1979; Neubig et al., 1979).

Changeux has found that solubilized membranes, devoid of the 43,000 protein, could be restored to a "functional" state such that the receptor can undergo discrete conformational changes in affinity states associated with agonist binding and that ion channel blockers still affect allosterically the binding of agonist to the receptor (Sobel et al., 1980; Heidmann et al., 1980). The alkaline extracted membranes bind the potent local anesthetic [ $^3$ H]-meprobamate and this binding is displaced by  $H_{12}$ -HTX as well as other local anesthetics (Neubig et al., 1979). The apparent affinity of  $H_{12}$ -HTX for the meprobamate site is identical to that previously characterized by the direct binding of [ $^3$ H]- $H_{12}$ -HTX to membranes (Eldefrawi et al., 1977; Elliot et al., 1977) suggesting that the binding of these ligands is mutually exclusive. Since the remaining proteins after removal of the 43,000 protein are composed almost entirely of nAChR subunits, it seems likely that the nAChR itself composes the ion conductance channel.

A basic assumption regarding the function of the nAChR-ICC complex is that ACh binding to the receptor induces, by an allosteric mechanism, a transition in the conformation of the channel. There are several reports in the literature consistent with this notion. For example, in nAChR enriched electroplax membranes, the interaction of compounds presumed to bind to the ICC is influenced allosterically by cholinergic

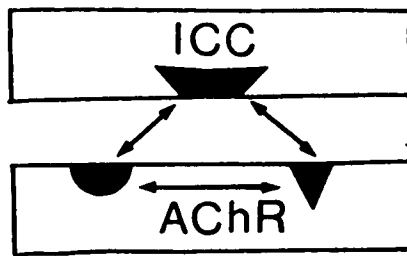
agonists. Binding of the radiolabelled local anesthetic [ $^3\text{H}$ ]-dimethisoquin is enhanced in the presence of carbachol (Cohen et al., 1978). Similarly, the measured fluorescence intensity resulting from the interaction of the local anesthetic quinacrine with torpedo membranes is increased in the presence of agonists as well as some antagonists (Grunhagen et al., 1976). Although it is not clear whether the enhanced fluorescence results from an increase in quantum yield or in total quinacrine bound, either possibility would depend on an allosteric interaction between the nAChR and the ICC. Recently, it has been shown that cholinergic agonists greatly enhance, by as much as several hundred-fold, the initial rate of binding of [ $^3\text{H}$ ]- $\text{H}_{11}$ -HTX to nAChR enriched torpedo membranes; receptor antagonists were found to block the agonist enhanced kinetics (Aronstam et al., 1981). These results are in agreement that the binding of a ligand to the nAChR may allosterically induce an experimentally detectable change in the channel conformation such that its drug binding characteristics are altered.

In a reciprocal manner, the binding of a ligand to the ICC might be expected to result in an allosteric effect on the nAChR such that the binding of receptor ligands might be altered. Indeed, using ethidium bromide fluorescence as a marker for the nAChR, Schimmerlik et al. (1979) have reported a slow conformational change in the receptor following the more rapid binding of HTX. Evidence that this HTX induced conformational change alters the agonist recognition site has been presented. In the presence of HTX or  $\text{H}_2$ -HTX, at concentrations in which these ligands were shown to inhibit the carbachol induced response, [ $^3\text{H}$ ]-acetylcholine binding to electroplax membranes has been found to be enhanced (Kato et al., 1976). Furthermore, the S-shaped binding curve

for [ $^3\text{H}$ ]-ACh normally having a Hill coefficient of 1.4 was converted to a hyperbolic one (Hill coefficient = 1.0) in the presence of these channel ligand drugs. A similar enhancement of [ $^3\text{H}$ ]-ACh binding and conversion of a sigmoidal binding curve to a hyperbolic one was observed in the presence of the local anesthetic prilocaine (Cohen et al., 1974). In a study of the kinetics of [ $^3\text{H}$ ]-ACh binding to electroplax membranes, under conditions where the half time for binding was 50 seconds, the addition of  $10\mu\text{M}$  of the local anesthetic dimethisoquin resulted in equilibrium of [ $^3\text{H}$ ]-ACh binding within 10 seconds of mixing (Neubig et al., 1979). These results provide direct evidence that the nAChR and the ICC are linked to one another in an indirect (allosteric) way. The binding of a ligand to one of these units and the concomitant conformational change in the polypeptides to which it binds can confer secondary conformational change in other polypeptide components of the complex which contain the binding sites for other ligands.

#### SPECIFIC ADMS

Based on the models of allosteric enzymes, the binding of a ligand to a site on one protein subunit of a macromolecular complex such as a receptor may induce an alteration in the conformation at some distant site and on a distinct subunit. Evidence obtained from the binding studies using electropex membranes discussed above strongly indicates that the nAChR- complex behaves as an allosteric protein. Additionally, there is evidence in electropex tissue for a conformational interaction between a distinct and physically separate binding site for agonists and one for antagonists on the nAChR which can account for the apparent competitive nature of these drugs. This is in contrast to the usually assumed mutually exclusive binding of agonists and antagonists in which they compete for a common site. The former hypothesis can account for experimental results which strongly suggest simultaneous occupancy of the receptor by both an agonist and an antagonist ligand such as a snake  $\alpha$ -toxin (Sarvey et al., 1978; Bulger and Hess, 1973; Fu et al., 1974; Hess et al., 1975). These same allosteric relationships may be present in skeletal muscle systems and may be detected by probes which are sensitive to conformational transitions of the binding protein. The following model is proposed to accommodate these concepts and to serve as a framework for this study. According to this model, the binding of a ligand to any one of the three sites, i.e. agonist, antagonist or ICC, may influence the other two sites via conformationally linked transitions of the polypeptide on which the ligand binding site resides (indicated by arrows).



The availability of ligands selective for each of these sites will provide the tools to study these induced conformational changes. A second selectively binding ligand is used to detect the induced change.

The present study of the nAChR-complex from frog skeletal muscle is an attempt to use selective ligand binding to detect such induced conformational changes. The ligands and their effects on neuromuscular transmission are characterized at the level of their physiological response (muscle tension) and on the molecular level by binding studies. This provides information on the functional nAChR complex of intact muscle and also the effects of ligand binding at one specific site on the properties of other sites. The study utilizes carbachol and dTC, prototypic reversibly binding ligands selective for the agonist and antagonist binding sites, respectively. PCP was chosen as a reversible ligand having selectivity for the ICC primarily because of the unavailability of HTX and that, unlike other available channel ligands, it has low activity as a local anesthetic. The possibility that PCP was a channel blocker was established by the initial physiological findings in this study and subsequently by electrophysiological characterization by others which confirm that PCP has the required selectivity for the channel (Tsai et al., 1979; Albuquerque et al., 1980). In addition to reversible ligands this study employs DPA, an alkylating agent which acts as an irreversible agonist on the frog rectus abdominis preparation

(Mittag and Tormay, 1970). Utilization of an irreversible agonist enables one to study the receptor-complex in which a ligand induced conformation has been "frozen" by the irreversible drug. In this case the agonist induced conformation is formed and can be studied with other ligands without the need to consider equilibria between conformational states occurring with two reversible ligands present at one time. An irreversibly acting ligand also affords the added advantage that the unbound drug is washed free from the muscle, minimizing possible non-selective interaction of the ligand with other binding sites on the nAChR-complex. Finally, [ $^{125}$ I]  $\alpha$ -BTX was selected for use as the detector of induced conformational changes in the nAChR-complex resulting from binding of small ligands of each of the three classes. The extremely high selectivity of  $\alpha$ -BTX binding for the receptor as well as its large size (M.W.=8000), making multipoint attachment likely, suggest that it may be sensitive to very subtle changes in conformation of the receptor subunits that could affect its binding parameters. In addition to the effect of previously mentioned ligands on toxin binding  $\alpha$ -BTX binding is also studied in chemically modified nAChR. The two types of modification included in this study are muscles that have been previously reduced with DTT and ones that have been reduced and alkylated with DPA. Since it is necessary to reduce the muscle in order to achieve the irreversible agonist activity of DPA, reduced receptors serve as the control for those that have been DPA alkylated. Results obtained from binding studies on receptors in situ in the intact muscle are contrasted with similar experiments performed on detergent solubilized nAChR preparations.

$\alpha$ -BTX binding studies are performed on solubilized and membrane

bound nAChR under pseudoequilibrium conditions, i.e. binding is allowed to proceed for a given fixed time interval which is determined on the basis of an apparent saturation of sites at this time. Although a true equilibrium is not obtained due to the extremely slow rate of toxin dissociation from the receptor, useful information can be obtained regarding the number and relative affinity of toxin binding sites after chemical modification of the receptor or in the presence of small competing ligands. A change in apparent affinity for toxin binding due to competition may be the result of a direct interaction with the toxin binding site or an indirect one mediated via conformational changes in the nAChR as a result of ligand binding at a distant site. A reduction in number of binding sites, without change in toxin binding affinity for the remaining sites, must clearly result from an indirect effect for a reversibly binding ligand. More detailed information will be obtained by kinetic analysis of the toxin binding reaction to solubilized but not intact nAChR (due to the slow rate of toxin diffusion through the muscle). Kinetic analysis permits differentiation of the action of effectors on one, or the other (or both) of the previously reported toxin binding sites.

## MATERIALS AND METHODS

### 1. Preparations

#### A. Detergent Solubilized Acetylcholine Receptor Preparation

Frogs were killed by decapitation followed by spinal pith. Whole sartorius muscles were removed bilaterally and were reduced, alkylated or used untreated. Soluble nAChR was prepared (in each case) from each type of sartorius muscle (treatment). For extraction of nAChR, muscles were minced in 1 ml of 1 M KBr per sartorius muscle (approx. 50-100 mg net wt.), homogenized by a Brinkman Polytron (setting 3 for 10 sec.) and centrifuged at 5000xg for 20 min. Membranes were resuspended in 1 ml of 1 M KBr per muscle homogenized and again centrifuged at 5000xg for 20 min. The pellet was extracted overnight at 4° C in 1 ml of 2% Triton X-100 per sartorius muscle used for the preparation. Following centrifugation at 27,000xg for 20 min. the supernatant was passed through a Millipore type AA membrane (.8 $\mu$  pore size) and made 10mM in NaPO<sub>4</sub> buffer (pH 7.4). Soluble receptor preparations were kept at 4°C and always used within 4 days of preparation.

#### B. Purification of $\alpha$ -Bungarotoxin

$\alpha$ -BTX, purchased as pure from Miami Serpentarium, was further purified by ion exchange chromatography. Two milligrams of freeze dried toxin were dissolved in 1.5 ml distilled H<sub>2</sub>O and applied to a 6 ml column of Sephadex SP-C25. After 30 minutes the column was washed with 25 ml of 25mM NaPO<sub>4</sub> buffer (pH 7.4).  $\alpha$ -BTX was eluted with 0.8ml fractions of 150mM NaCl. The concentration of  $\alpha$ -BTX in the collected fractions was determined spectrophotometrically (E=8300 at 273nm). Solutions of the purified toxin were stored at -20°C.

### C. Radioiodination of $\alpha$ -BTX

Incorporation of  $^{125}\text{I}$  into  $\alpha$ -BTX was accomplished utilizing either the solid state lactoperoxidase (David, 1972) or Iodogen method (Fraker et al., 1979); the latter generally gave the higher specific activity and was used for the major portion of this work. The method of purification of [ $^{125}\text{I}$ ]  $\alpha$ -BTX following either method of incorporation was identical.

Lactoperoxidase method. A 50% suspension of lactoperoxidase coupled to Sepharose 2B (Worthington Biochemicals) was washed twice with a 40-fold excess of 150mM NaCl -- 200mM NaPO<sub>4</sub> buffer (pH 6.5). Washed solid phase lactoperoxidase (50 $\mu$ l) was added to 5mCi carrier free Na  $^{125}\text{I}$  (New England Nuclear). Five nanomoles of repurified  $\alpha$ -BTX was added at a concentration of  $10^{-6}$ - $10^{-5}$ M. The reaction was initiated by addition of a total of 12.5 nanomoles of H<sub>2</sub>O<sub>2</sub> in five equal aliquots at ten min. intervals. The reaction vial was gently rotated by hand over the 50 min. reaction period.

Iodogen method. A fresh solution of 0.2mg/ml Iodogen (1,3,4,6-tetrachloro-3,6-diphenylglycouril; Pierce) was prepared in chloroform. Ten microliters of this solution was added to a small glass test tube (6x50mM) which was then rotated at a 45° angle under a slow stream of dry N<sub>2</sub> until chloroform evaporation was complete. To this tube was added 5 nmoles of repurified  $\alpha$ -BTX (40  $\mu$ g), 10 $\mu$ l of 100mM NaPO<sub>4</sub> (pH 7.5) and 5 mCi carrier free Na  $^{125}\text{I}$  (NEN). The tube was mixed by gentle rotation and set on ice for 60 min. Since the Iodogen which coats the walls of the reaction tube is virtually insoluble in aqueous systems, the oxidation reaction occurs at the coated glass surface.

Purification of [ $^{125}$ I]  $\alpha$ -BTX. Quantitative transfer of the contents of the reaction vial to a cascade system consisting of a 1 ml column of QAE-A25 Sephadex (chloride form) over a 6 ml column of SP-C25 Sephadex (sodium form) was effected using 2 ml of distilled  $H_2O$ . The role of the QAE, a strongly basic ion exchanger, is the adsorption of unreacted  $^{125}I$  as well as the removal of traces of the solid phase iodogen or beaded lactoperoxidase.  $\alpha$ -BTX, which has a positive charge under the conditions employed, passes freely through the QAE column and is adsorbed onto the SP column (a strongly acidic cation exchanger). The SP-C25 column was washed with 25 ml of 25mM Na/PO<sub>4</sub> buffer (pH 7.2) to elute oxidation products of the iodination reaction as well as other possible contaminants. Iodinated  $\alpha$ -BTX was desorbed by addition of 0.8ml aliquots of 150 mM NaCl. The 2 or 3 fractions containing the highest concentrations of radioactivity were pooled and specific activity was assessed by the isotope dilution method with cold  $\alpha$ -BTX. Typically, the lactoperoxidase method yielded a specific activity of 150-300 cpm/fmole while the iodogen method yielded  $I^{125}$   $\alpha$ -BTX about twice this specific activity. Radioactive toxin was stored at 4°C for up to one  $^{125}I$  half life (60 days) before repurification on SP-C25 as described here.

D. Preparation of N,N-dimethyl-2-phenyl aziridinium (DPA)

DPA is spontaneously formed by cyclization of N,N-dimethyl-2-chloro-2-phenylethylamine in aqueous solution at pH 7.0. It has previously been shown that the cyclization to produce DPA is complete in less than 1 min. at 23°C in pH 7.4 buffer (Chapman et al., 1963). Synthesis of N,N-dimethyl-2-chloro-2-phenylethylamine was achieved by prior synthesis of the alcohol precursor N,N-dimethyl-2-hydroxy-2-phenylethylamine as

previously described (Chapman, et al., 1963) followed by chlorination.

N,N-dimethyl-2-hydroxy-2-phenylethylamine. To 1 mole of anhydrous dimethylamine dissolved in 200 ml dry benzene was added 0.4 moles of 1,2-epoxyethylbenzene. The reaction was allowed to proceed for 10 days at room temperature. The product was collected by fractional distillation in approximately 80% yield (B.P.=135-140/18mm Hg).

N,N-dimethyl-2-chloro-2-phenylethylamine. Thionyl chloride, at slight excess (5%) was allowed to react with N,N-dimethyl-2-hydroxy-2-phenylethylamine in dry chloroform at 0°C for 3 hrs. The mixture was evaporated to dryness in vacuo and the residue recrystallized three times from ethanol-methanol. The product was obtained at 35% yield and its melting point was 204-206°C. The product obtained was compared to a reference sample of purified material (Rosen et al., 1971) by the mixed melting point method.

## 2. Experimental

### A. Isolated Frog Hemi-Rectus Abdominis Preparation.

The rectus abdominis muscle from 3 1/2-5 inch Rana pipiens was excised and set up in a 10 ml organ bath as previously described (Livingstone, et al., 1968). Muscles were preloaded with 1 gram of tension and continuously aerated in frog Ringer's solution at 22°C. Composition of the Ringer's solution in meqs per liter was as follows:  $\text{Na}^+$ , 113.7;  $\text{K}^+$ , 1.9;  $\text{Ca}^{++}$ , 2.2;  $\text{Cl}^-$ , 115.3;  $\text{H}_2\text{PO}_4^-$ , .1;  $\text{HCO}_3^-$ , 2.4; and glucose, 10mM. Bathing solutions were changed by draining and refilling the bath. Dose response curves to carbachol were done cumulatively (unless otherwise noted) allowing 4 minutes to reach equilibrium for a given dose. Isometric contractions were recorded on a Grass Model 7 polygraph using a Grass FT03C force displacement transducer.

#### B. Con A Column Binding Assay.

The high affinity that detergent solubilized nicotinic AChR has for concanavalin A (Con A) is the basis of a very useful assay for nAChR labelled by  $\alpha$ -BTX (Mittag, et al., 1976; Mittag, et al., 1978). Con A coupled to Sepharose 2B is mixed with Sepharose 2B gel in a ratio of 1:5 by volume. Disposable plastic columns were prepared containing 1 ml of gel and washed with 5 ml of buffer containing 150 mM NaCl, 5mM NaPO<sub>4</sub> (pH 7.4) and 1% Triton X-100 (PBS/1%). A sample containing solubilized nAChR complexed to labelled  $\alpha$ -BTX in a volume of 100-250 $\mu$ l was applied to the gel and allowed to equilibrate for 15 min. at 4°C. The columns were washed free of noncomplexed labelled  $\alpha$ -BTX with 2-5 ml aliquots of PBS/1%. [<sup>125</sup>I]  $\alpha$ -BTX bound to receptors was retained on the column and was determined by directly counting the residual radioactivity bound to the Con A columns in a Beckman Biogamma 2 gamma counter.

#### C. Binding of [<sup>125</sup>I] $\alpha$ -BTX to solubilized nAChR: Apparent Equilibrium method.

Triton solubilized nAChR (350 $\mu$ l) was incubated with the desired concentration of radiolabelled  $\alpha$ -BTX for 24 hrs at 4°C. Reaction volumes were kept constant (500 $\mu$ l) by addition of 150mM NaCl. Aliquots from each sample were assayed for receptor-toxin complexes on Con A columns in duplicate. Non-specific binding was determined by either adding a 50-fold excess of unlabelled  $\alpha$ -BTX or 0.1mM dTC at 4°C 1 hr prior to addition of labelled  $\alpha$ -BTX. Similar results were obtained using either ligand for determination of non-specific binding.

#### D. Competition by Unlabelled Ligands for [<sup>125</sup>I] $\alpha$ -BTX to Solubilized nAChR.

Prior to addition of labelled  $\alpha$ -BTX to solubilized nAChR the

competing ligand (carbachol, dTC, or PCP) was added to samples in the concentration range of  $10^{-7}$ - $10^{-1}$  M and incubated for 1 hr at 4°C (reaction volumes were brought to 0.5ml with distilled H<sub>2</sub>O). Labelled  $\alpha$ -BTX was added to give a concentration of 5nM (approx. 2-fold the apparent K<sub>d</sub> determined with toxin alone under the same conditions) and the binding reaction was allowed to proceed for either 180min. or 24 hrs ("long" incubation experiments) at 4°C. Aliquots of each sample were assayed in duplicate for nAChR- $\alpha$ -BTX complexes on Con A columns. Non-specific binding was determined as above using a 50-fold excess of labelled over unlabelled  $\alpha$ -BTX.

E. Binding of [<sup>125</sup>I]  $\alpha$ -BTX to Whole Frog Sartorius Muscle:

Apparent Equilibrium Method.

Intact sartorius muscles were removed from *Rana pipiens* and placed in ice cold amphibian Ringer's solution. After dissection was complete, groups of 3 muscles (randomly selected) were placed in individual vials containing 1.5 ml of Ringer's solution and the desired concentration of labelled  $\alpha$ -BTX ( $10^{-10}$ - $10^{-7}$  M). After a 40 hr. incubation at 4°C the intact muscles were washed free of unbound  $\alpha$ -BTX. This was accomplished by removal of each trio of muscles to a vial containing 20 ml of ice cold Ringer's containing  $10^{-8}$  M in dTC. Fresh medium was replaced after 2 min. equilibration and this wash process was repeated 4 additional times. Muscles were blotted dry and finely minced with a scissors. Each mince, representing 3 individual muscles, was added to a glass homogenization tube containing 1.5 ml 2% Triton X-100 and homogenized with a motor driven teflon pestle. After a 1 hr extraction at 4°C, samples were centrifuged in a Beckman minifuge for 3 min. Receptor-toxin complex formation was measured in duplicate by the Con A column

assay of an aliquot of the supernatant. Total nAChR present was evaluated by relabelling an aliquot of each supernatant with  $2 \times 10^{-8}$  M [ $^{125}$ I]  $\alpha$ -BTX. Non-specific binding was determined by incubation of muscle trios with a 20-50 fold excess of unlabelled  $\alpha$ -BTX for 2 hrs at  $4^{\circ}\text{C}$  prior to addition of labelled toxin.

F. Competition by Unlabelled Ligands for [ $^{125}$ I]  $\alpha$ -BTX Binding to Whole Sartorius Muscle

The protocol for these experiments was as above with the following exceptions. Prior to the 40 hr. toxin labelling, muscles were preincubated for 8hrs at  $4^{\circ}\text{C}$  with the desired concentration of competitor (carbachol, dTC, or PCP). All bindings were performed using a 3nM concentration of [ $^{125}$ I]  $\alpha$ -BTX. Non-specific binding was determined by simultaneous addition of a 33-fold excess of cold toxin ( $1 \times 10^{-7}$  M).

G. Association Rate Measurements of [ $^{125}$ I]  $\alpha$ -BTX Binding to Solubilized nAChR

Triton solubilized receptor preparations (3.5 ml) were brought to a final volume of 4 ml with distilled  $\text{H}_2\text{O}$  and/or ligands competing for  $\alpha$ -BTX binding (carbachol, dTC, or PCP). Competing ligands, if present, were allowed to equilibrate with the receptor for 1 hr at  $4^{\circ}\text{C}$  prior to toxin addition. Initiation of the binding reaction was by the rapid addition of [ $^{125}$ I]  $\alpha$ -BTX to a final concentration of 2.6nM with vigorous vortexing. The binding reaction was stopped at the desired time interval (5-3000 min) by withdrawal of an aliquot and addition to a vial containing a 50-100 fold excess of unlabelled  $\alpha$ -BTX. Complex formation was determined in duplicate at each of 15 time points by the Con A column assay. Non-specific binding was measured by addition of a 20-50

fold excess of cold toxin prior to addition of labelled toxin.

#### H. Dissociation Rate Measurements of [ $^{125}$ I] $\alpha$ -BTX Complexed to Detergent Extracted nAChR

Labelled receptor-toxin complexes were prepared by overnight labelling of detergent solubilized nAChR with  $1 \times 10^{-8}$  M [ $^{125}$ I]  $\alpha$ -BTX. Dissociation of complexes was initiated by introduction of a 50-fold excess of unlabelled  $\alpha$ -BTX ( $5 \times 10^{-7}$  M final concentration). At the desired times over the course of 72 hrs complex concentration was assessed by the Con A column assay. To control for enzymatic loss of receptor-toxin complexes and receptor denaturation, samples in which there was no addition of unlabelled toxin were subjected to the Con A assay over the time course of the experiment. Non-specific binding was measured by assaying receptors that were preincubated with a 50-fold excess of unlabelled  $\alpha$ -BTX 1 hour prior to addition of label.

#### I. DTT Reduction of Whole Sartorius Muscle.

Freshly dissected whole sartorius muscles were incubated in 2mM DTT in amphibian Ringer's solution for 15 min. at 22°C. Muscles were washed free of DTT by replacement with fresh medium (50 ml amphibian Ringer's; 22°C) five times and then blotted dry. DTT treated muscles were substituted for naive muscle in experiments designed to study [ $^{125}$ I]  $\alpha$ -BTX binding to receptors which had been reduced by DTT in the intact muscle in parallel with the studies done on muscle bath tissues.

#### J. DPA Alkylation of Whole Sartorius Muscle.

Intact sartorius muscles were reduced with 2mM DTT and washed as above (section I.). Muscles were then immersed in a freshly prepared solution (prepared within 2 min of use) of  $5 \times 10^{-4}$  M DPA and incubated for 30 min at 22°C. The incubation medium bathing the muscles was replaced

5 times with fresh medium (50ml amphibian Ringer's, 22°C) to wash away excess DPA. The muscles were then blotted dry and compared to naive muscle by using the binding of  $\alpha$ -BTX to whole muscle as described in section E. Alternatively, the muscle was used for preparation of DPA alkylated soluble nAChR.

### 3. Data analysis

#### A. Apparent Equilibrium Binding of [ $^{125}$ I] $\alpha$ -BTX to nAChR.

Pseudoequilibrium binding data was analyzed using the PROPHET<sup>1</sup> computer program "Binding." The program evaluates both the  $K_D$  apparent and the total number of toxin binding sites present ( $B_{max}$ ) and plots the data by hyperbolic and double reciprocal methods. The data were evaluated from a fit to the hyperbolic binding equation:

$$Y = \frac{1}{1 + \frac{K_D}{A}}$$

Where Y = concentration of toxin-receptor complex formed in the presence of  $\alpha$ -BTX concentration A.

$K_D$  = apparent dissociation constant of  $\alpha$ -BTX for binding to the receptor.

A = concentration of  $\alpha$ -BTX.

The parameters Y,  $K_D$ , and  $B_{max}$  were generated by the iterative fitting procedure. The results were also analyzed by the method of Scatchard (1949) and values for  $K_D$  and  $B_{max}$  obtained were similar to those obtained by non-linear regression analysis using the PROPHET system.

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1. The PROPHET system is a national computer resource sponsored by the National Institutes of Health.

B. Dissociation kinetics of [ $^{125}$ I]  $\alpha$ -BTX from [ $^{125}$ I]  $\alpha$ -BTX-AChR complexes.

The rate of dissociation of [ $^{125}$ I]  $\alpha$ -BTX from the nAChR was determined using the PROPHET curve fitting program "Expfit." The Expfit program fits data to the exponential decay equation:

$$Y = Ae^{-kT}$$

Where Y = concentration of toxin-receptor complex at time T  
 T = time (minutes) after dilution of reaction mixture with a large excess of cold toxin.  
 A = initial concentration of receptor-toxin complex.  
 k = dissociation rate constant of receptor-toxin complex.

Assuming a homogeneous population of receptors and given values for Y at time T, the program calculates the initial concentration of complex (A) and the dissociation rate constant (k). It also fits a regression line to the data and calculates the correlation coefficient for that fit.

C. Kinetics of association of [ $^{125}$ I]  $\alpha$ -BTX to nAChR.

The data were analyzed assuming that either a single kinetic component or that two distinct and independent kinetic components were present for the toxin binding reaction. The equations used were the integrated second order rate equations for toxin-complex formation as a function of time and free toxin concentration. Dissociation of complex is negligible on this time scale and is ignored.  
 one component:

$$\text{BTX-AChR (T,B)} = A_0 \left( 1 - \frac{B_0 e^{-k T(B_0 - A_0)}}{B_0} \right)$$

two component:

$$\text{BTX-AChR } (T, B) = A_0 \left( 1 - \frac{B e^{-k_1 T (B_0 - A_0)}}{B_0} \right) + C_0 \left( 1 - \frac{B e^{-k_2 T (B_0 - C_0)}}{B_0} \right)$$

$A_0$  = total concentration of AChR (one component system) or initial concentration of AChR<sub>1</sub> (two component system).

$C_0$  = initial concentration of AChR<sub>2</sub> (two component system)

$B_0$  = initial concentration of  $\alpha$ -BTX

$B$  = concentration of  $\alpha$ -BTX at time  $T$

$T$  = time

$k_1$  = rate constant for association of  $\alpha$ -BTX to total AChR (one component system) or to AChR<sub>1</sub> (two component system)

$k_2$  = rate constant for association of  $\alpha$ -BTX to AChR<sub>2</sub>

Calculations were made using MLAB, a function of the PROPHET computer system, which performs multivariable curve fitting and nonlinear regression analysis. The values of  $A_0$  and  $k_1$  were solved for assuming a one component system. For the two component model four variables were solved for ( $A_0$ ,  $C_0$ ,  $k_1$ ,  $k_2$ ) making no further assumptions about the system.

## RESULTS

1. Formation and hydrolysis of DPA.

Since DPA is generated in solution from a 2-chloroethylamine precursor, the formation and hydrolysis of DPA was studied by proton NMR analysis of D<sub>2</sub>O solutions of N,N-dimethyl-2-chloro-2-phenylethylamine (preparation described in the Materials and Methods section). As shown in fig. 1, the resultant NMR spectrum is consistent with a rapid conversion to the aziridinium ion ring structure of DPA (see figure legend for details). Cyclization is complete within minutes at approximately 30°C. Once formed, the  $T^{1/2}$  for hydrolysis of the aziridinium ring of DPA in D<sub>2</sub>O was measured by Mittag (unpublished communication) to be 63 min. at 30°C at pH 7.2. As a consequence of the limited stability of the DPA ion, all solutions were made immediately prior to their use and muscle treatment with solutions of DPA never exceeded 30 minutes. This was done to minimize ambiguity in the experimental results due to the falling concentration of DPA and the increasing amount of the stable hydrolysis product of DPA (see scheme in the inset to fig. 1).

2. Effect of DTT reduction on carbachol induced muscle contraction.

Cumulative dose response curves were generated from measurements of carbachol induced tension in the frog hemi-rectus abdominis muscle preparation with and without prior reduction of the muscle with DTT (fig. 2). Treatment with 2mM DTT for 20 min. (23°C) resulted in a parallel shift to the right in the log dose response curve for carbachol (approx.  $1/2$  log unit) without a reduction in the maximal response obtained. The EC<sub>50</sub> for carbachol increased from  $1.86 \pm 0.16 \mu\text{M}$  (n=10) to  $6.83 \pm 0.53 \mu\text{M}$  (n=6) following DTT reduction of the muscle; both dose

Figure 1. Proton NMR spectrum of the cyclized product formed by N,N-dimethyl-2-chloro-2-phenylethylamine approximately 5 minutes after dissolution in D<sub>2</sub>O. Data points were gathered (4096) with an acquisition time of 0.511 sec on Varian FT 80A NMR. The spectrum is consistent with results obtained for a reference sample of known purity. It was determined that rapid cyclization to the aziridinium ion occurs (within 5 minutes) which is followed by a slower ring hydrolysis ( $T^{1/2} = 63$  min at 30°C). Integration of the area under peaks numbered 1, 2, 3 and 4 indicates a ratio of 11:2:12:4. Peak 1 is suggested to result from the aromatic protons and Peak 3 the methyl protons of DPA. Peaks 2 and 4 are thought to represent the C<sub>2</sub> and C<sub>1</sub> protons of DPA, respectively. Hydrolysis of the aziridinium ring in DPA should result in an upfield shift in the position of the methyl protons due to a loss of positive charge on the neighboring nitrogen atom. Peak number 5, therefore, is suggested to result from the methyl protons of the alcoholic hydrolysis product of DPA.

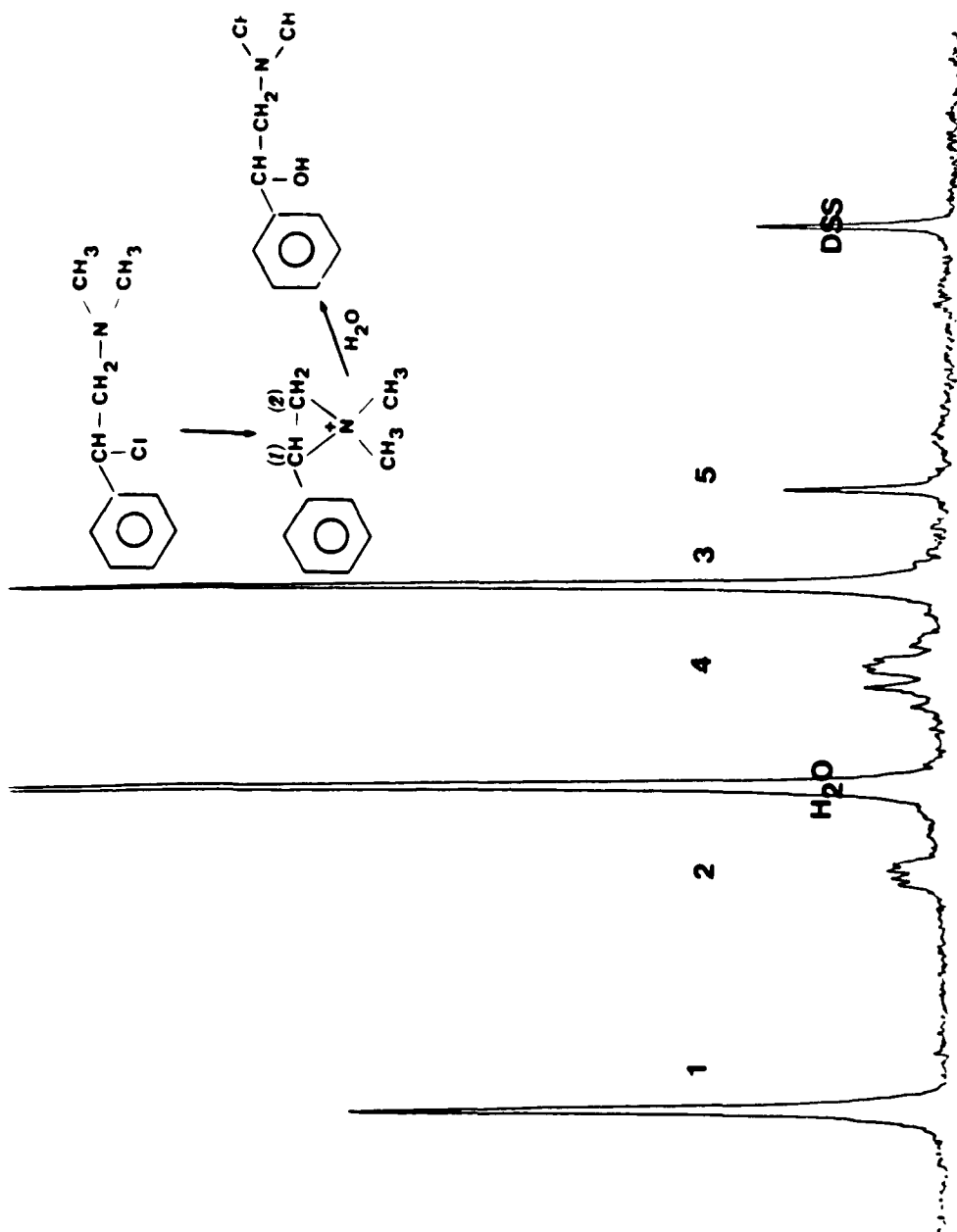
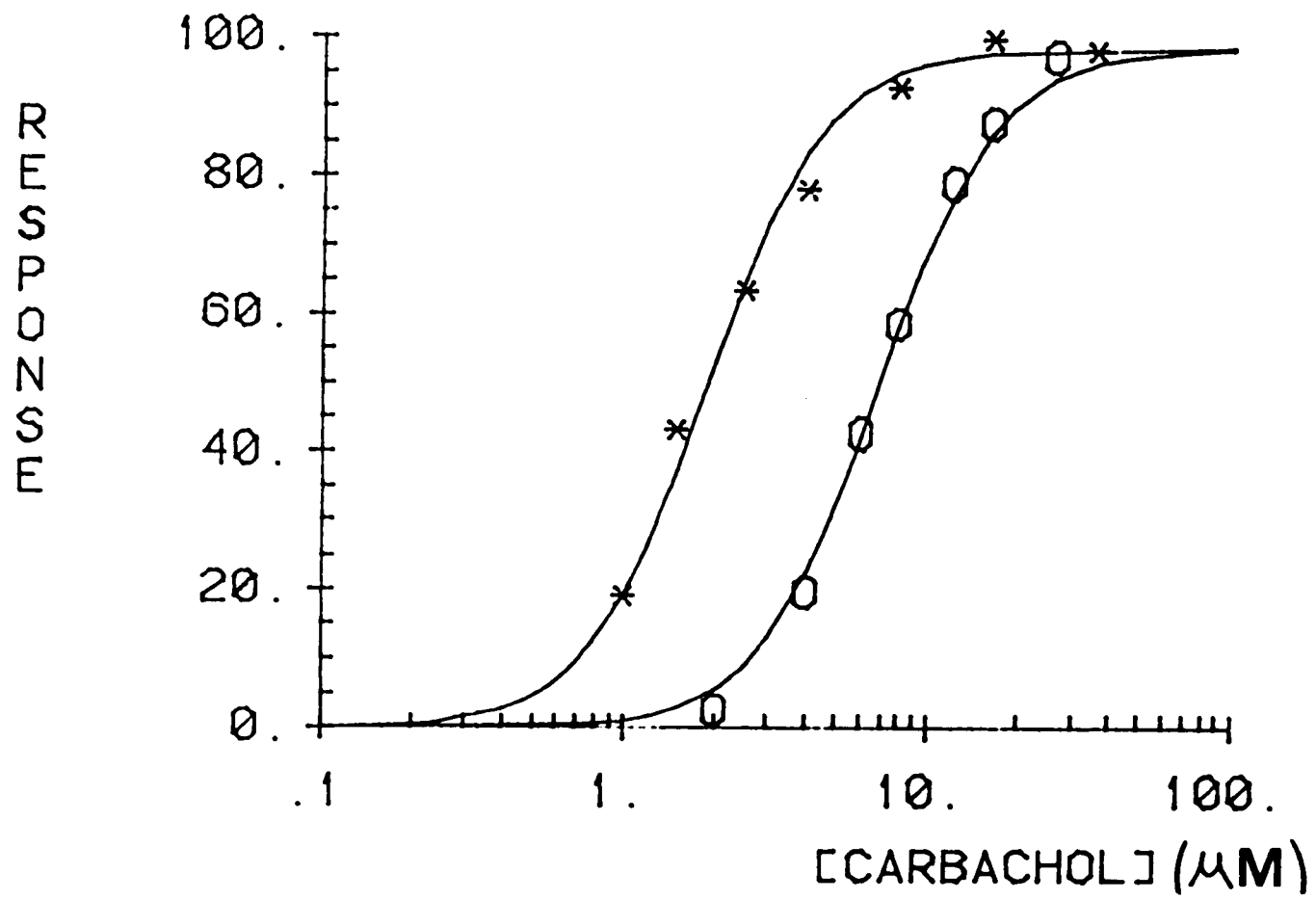


Figure 2. Effect of DTT reduction of muscle on carbachol induced tension response in frog rectus abdominis muscle preparation. Cumulative dose response curves were with 5 min time intervals between successive increases in agonist concentration. Response to carbachol is determined in muscles pretreated with 2mM DTT for 20 min at 23°C (\*) and in untreated preparation (0). Each data point represents the average response obtained for 6 and 10 individual muscle preparations, respectively. The average standard deviation of each of the data points was 4.75% with DTT treatment and 5.7% for control. A maximal response was still obtained after DTT treatment with an increase of  $EC_{50}$  to 6.83 $\mu$ M from 1.86 $\mu$ M observed in control preparations. The variance ratio test for nonparallelism (See Materials and Methods; Data Analysis) proved negative, indicating a common Hill Coefficient of 2.26 in these experiments.



response curves have a common Hill coefficient of 2.26. These results are comparable to the decrease in agonist affinity following DTT reduction as reported by other groups (Bleeham et al., 1980; Fleisch et al., 1974; Mittag et al., 1971).

### 3. Effect of DTT reduction on DPA induced muscle contraction.

DPA acts as a reversible agonist on the naive (no pretreatment) rectus abdominis preparation, attaining  $91.9 \pm 2.7\%$  of the maximum response produced by carbachol (fig. 3). The  $EC_{50}$  for DPA induced muscle contraction is  $17.0 \mu M$  ( $n=3$ ) with a Hill coefficient of  $1.61 \pm .15$ .

Contractions elicited by DPA are mediated by nAChR receptors. Selective blockade of acetylcholine receptors with  $\alpha$ -BTX treatment causes inhibition of both carbachol and DPA responses. Treatment of muscle preparations with  $1.3 \times 10^{-7} M$   $\alpha$ -BTX causes a time dependent reduction in responsiveness to test doses of  $25 \mu M$  carbachol; half maximal inhibition is observed after  $14.0 \pm 4.4$  minutes ( $n=4$ ), and complete block of this carbachol dose is seen after 30 minutes. After treatment with  $\alpha$ -BTX giving complete blockade to carbachol, the muscle is viable and fully responsive to contraction induced by  $30 mM$  KCl. Preincubation of rectus muscle for 30 minutes with  $1.3 \times 10^{-7} M$   $\alpha$ -BTX produced greater than a 95% reduction in the response to  $72 \mu M$  DPA ( $n=4$ ).

In common with carbachol, the immediate direct response to a given dose of DPA is diminished by prior DTT reduction of the muscle (fig. 4). However, the DPA contracture in reduced muscle, in contrast to naive muscle, is only partially reversed by extensive washing. Because of this "irreversible" agonist component a numerical evaluation of increase in  $EC_{50}$  due to DTT treatment could not easily be determined. Figure 4 shows a tracing of a physiograph record which illustrates the complete

Figure 3. Dose-response relationship for DPA on rectus abdominis muscle preparation. Cumulative doses of DPA were added to the muscle bath at 5 min intervals. Each data point represents the average response obtained by 3 individual muscle preparations (mean std. deviation for the six sets of data pts. was 2.7%). The maximum response obtained was  $91.9 \pm 2.7\%$  of the maximum carbachol induced response ( $ED_{50} = 17.0 \mu M$  DPA, Hill coefficient =  $1.6 \pm .15$ ).

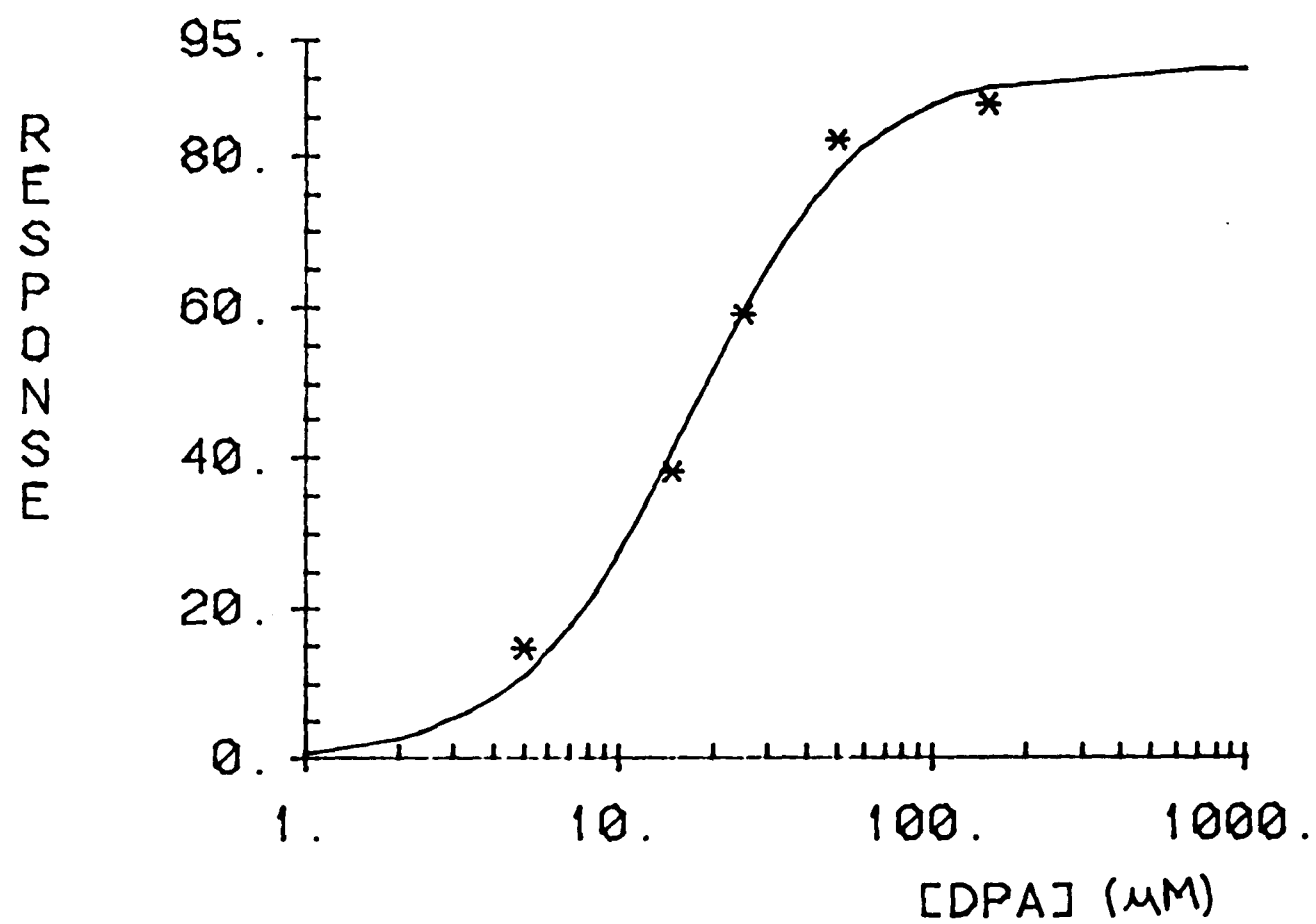
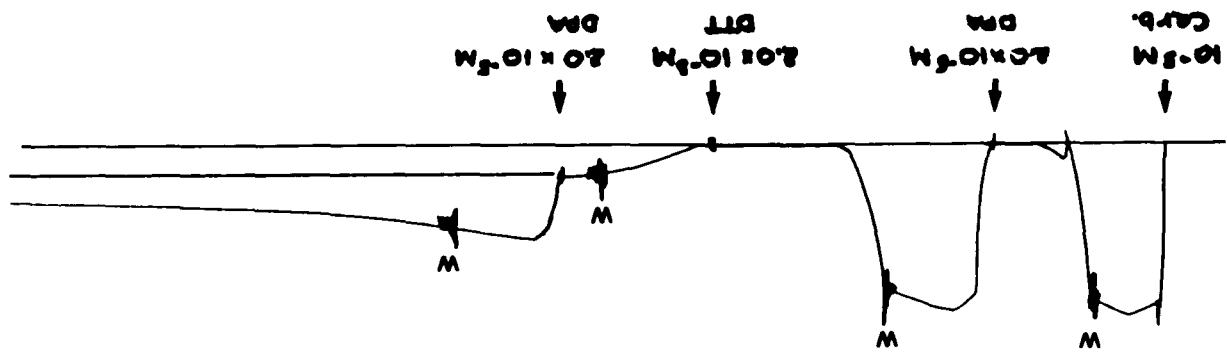


Figure 4. The irreversible component of DPA agonism obtained on muscle previously reduced with DTT. This muscle tension trace indicates that following 2mM DTT treatment for 20 min, the immediate DPA response is reduced in magnitude compared to the same DPA dose in nonreduced preparations. It also is no longer rapidly reversed by drug washout. The small increase in baseline tension following DTT treatment alone which is seen in this trace is variable in occurrence and has little effect on the maximal response which can be elicited by carbachol (see previous fig.).



Ib.

reversibility of the normal DPA response which is changed by DTT pretreatment of the muscle into a partially reversible response with a substantial irreversible component. The component of muscle tension which is not reversed within 30 minutes of drug washout is extremely stable, with less than 10% tension decline in the next 200 minutes (12 preparations). The origin of the "irreversible" component of DPA action is consistent with sulfhydryl group alkylation by the reactive aziridinium ion (Mittag and Tormay, 1970).

#### 4. "Irreversible" DPA contracture-protection experiments.

Ligands of each of the three functional classes known to interact with the nAChR complex (agonists, antagonists and channel blockers) were tested for their ability to protect against the sustained contracture resulting from DPA treatment of DTT-reduced muscle. The criteria for protection was the absence or reversal of the "irreversible" tension component after prolonged washout of the muscle treated with DPA in the presence of the test (protecting) ligand. By these criteria, protection was observed with ligands of each of the three classes of ligand.

A typical physiograph record demonstrating the ability of carbachol to protect against the sustained DPA response is shown in fig. 5. Panel A shows the tension response of a muscle previously reduced by DTT which has been treated with 1mM carbachol prior to and during exposure to 0.5mM DPA. Unlike the control (panel B), the muscle treated with carbachol displays no residual tension following washout of DPA and carbachol. Subsequent to treatment described in panel A the muscle is fully responsive to cholinergic agonists (not shown in record).

Protection against active site alkylation by DPA was also afforded by dTC treatment (fig. 6). Addition of 0.25 mM DPA to reduced muscle

Figure 5. Protection of rectus abdominis muscle by carbachol from the irreversible component of DPA action. In panel B the figure shows the time course of tension development in a muscle previously reduced with DTT (2mM-20 mins, 23°C) in response to 0.5mM DPA addition for 20 min i.e. non-protected. In panel A the DPA was administered to DTT reduced muscle preincubated with 1mM carbachol for 10 min. DPA treatment in the presence of carbachol, in contrast to non-protected muscle, results in no sustained contracture following washout of the reversibly bound DPA.

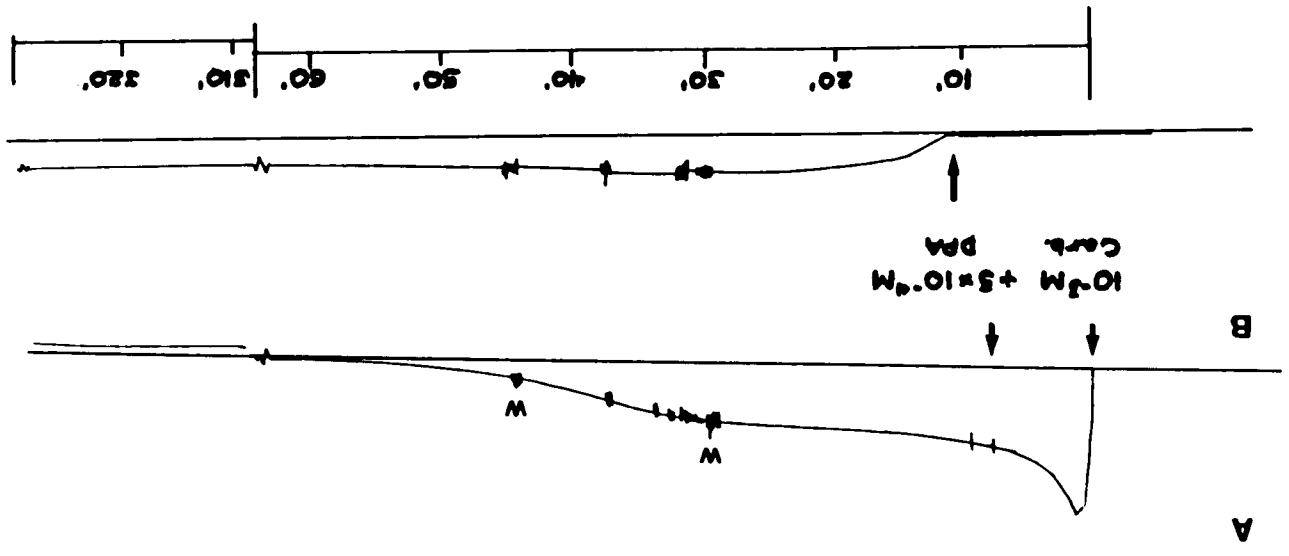
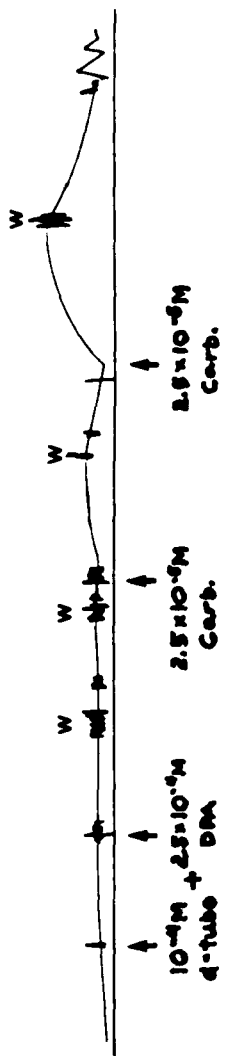
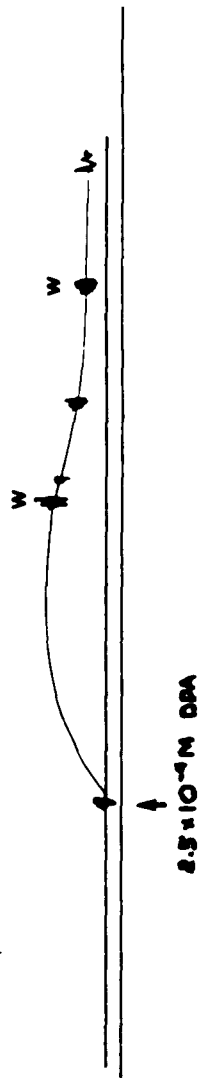
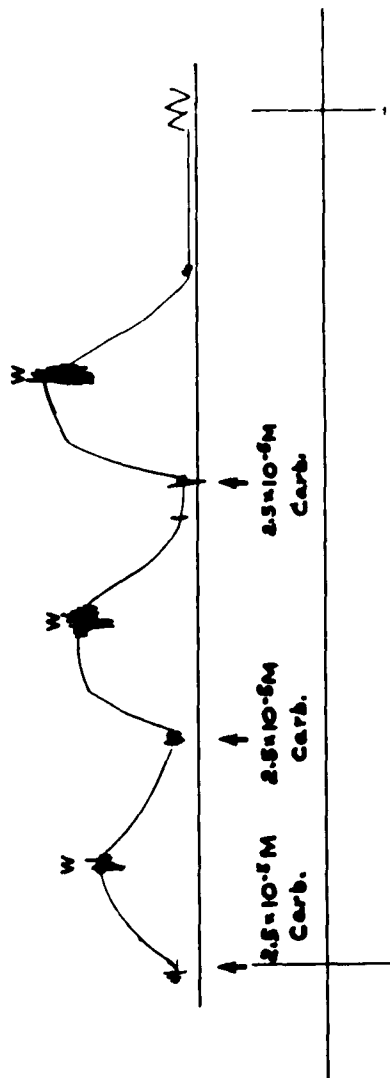


Figure 6. Protection of rectus abdominis muscle by dTC from the irreversible component of DPA action. The figure shows a continuous tension tracing of a muscle which was previously reduced with DTT (2mM-20 min). Pretreatment with 0.1mM dTC for 10 min results in no response to 0.25 mM DPA. After 20 min in the combined presence of DPA and dTC the drugs were washed out. Repeated test doses of carbachol show a slow recovery for the dTC inhibition. When the muscle has almost completely recovered from the protective dose of dTC, no sustained DPA contracture is observed. At this time addition of DPA alone (0.25mM-20 min) results in a response which is similar to the normal response of reduced muscle to DPA, i.e., it is only partially reversed following drug washout. However, the level of irreversible DPA contracture under these circumstances is less than usual. This may be attributed to some reoxidation of reduced muscle resulting from the long time elapsed since the initial DTT reduction (200 min).



75min



which has been pretreated with 0.1mM dTC results in no observed tension response. At a time when the direct dTC inhibition has reversed by washing, as judged by repeated test doses of agonist (25 $\mu$ M carbachol), no residual DPA induced tension is observed. That the muscle is functional following this treatment, and still at least partially reduced, is indicated by a responsiveness to a second dose of DPA that is incompletely reversed by washing.

In common with dTC, PCP is an inhibitor of both the reversible and "irreversible" agonist action of DPA. Physiograph records of tension response in control (panel A) and PCP treated muscle (panel B and B<sup>1</sup>) are shown in fig. 7. Pretreatment of the muscle with PCP (25 $\mu$ M-60 min.) reduced the muscle responsiveness to DPA (0.25mM) to less than 10% of control values in each of four preparations. After allowing PCP to dissociate for an excess of 19 hrs there was no increase in muscle tension, indicating DPA alkylation was prevented by PCP. However, after extensive washout the muscle is capable of activation and alkylation by a second treatment with DPA alone (panel B<sup>1</sup>).

##### 5. DPA induced contracture - Antagonism of response by drugs.

Antagonism of the sustained contracture resulting from DPA treatment occurred with the presumed channel blocking ligands PCP (fig. 8) and adiphenine (not shown). Complete inhibition of the DPA contracture was observed following addition of 25 $\mu$ M PCP to the bath, with half maximal effect attained after 18.5 $\pm$ 2.8 minutes of incubation. After tension has been reverted by PCP a maximal response is still observed as a result of K<sup>+</sup> induced depolarization (30mM KCl) confirming contractile viability. A concentration dependence is observed for the rate at which PCP reverses the DPA induced contracture (Table 1). Restoration of con-

Figure 7. Protection of rectus abdominis muscle from the irreversible component of DPA action. Muscle tension tracings are shown for control non-protected (panel A) and for PCP protected muscle (panel B, contd. in B<sup>1</sup>) in response to DPA treatment. In contrast to control muscle, pretreatment with 0.25mM PCP for 60 min results in virtually no response to a dose of 0.25mM DPA. After incubation with DPA for 20 min. followed by a prolonged washout (greater than 19 hrs), the PCP pretreated muscle, unlike control, displays no residual tension. After washout from the PCP pretreated muscle the response to DPA displays the normal irreversible component (panel B<sup>1</sup>).

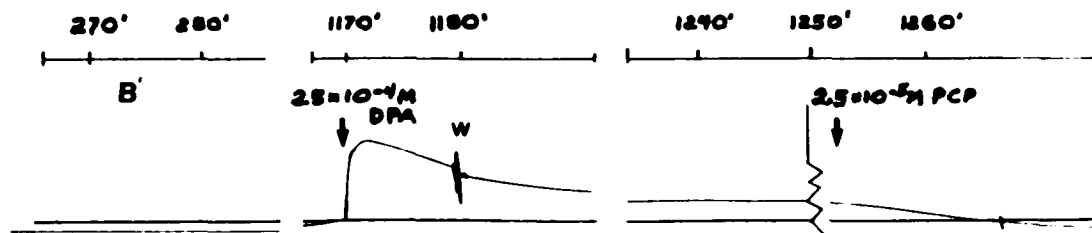
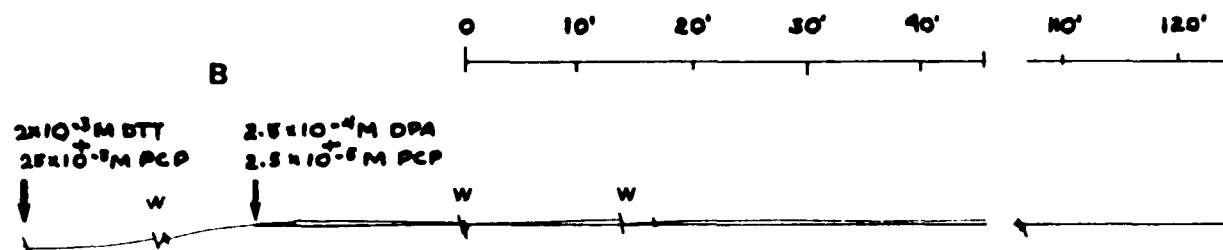
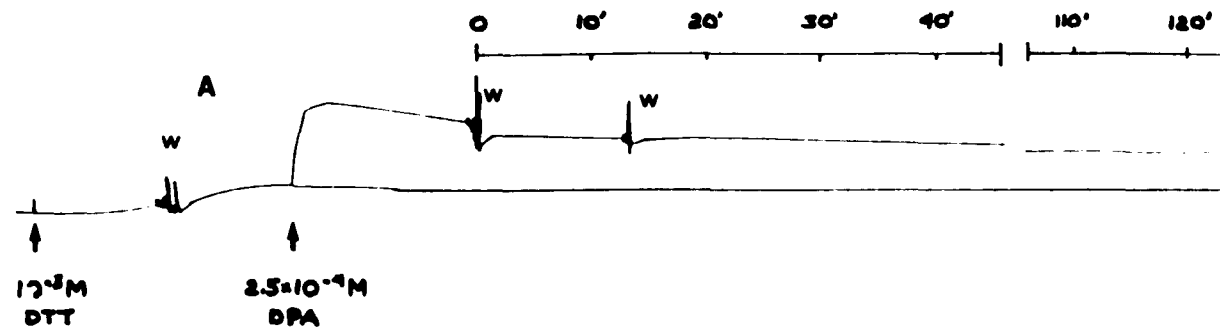


Figure 8. Blockade of the sustained DPA contracture of rectus abdominis muscle by PCP. A sustained muscle contracture is obtained by reduction of the muscle with DTT (2mM-20 min) followed by addition of 0.25mM DPA for 20 min and drug washout. This "irreversible" component of DPA action is blocked by 25 $\mu$ M PCP ( $T^{1/2}$  = 18.5 $\pm$ 2.8 min). PCP is shown not to inhibit muscular contraction induced by 30 mM KCl.

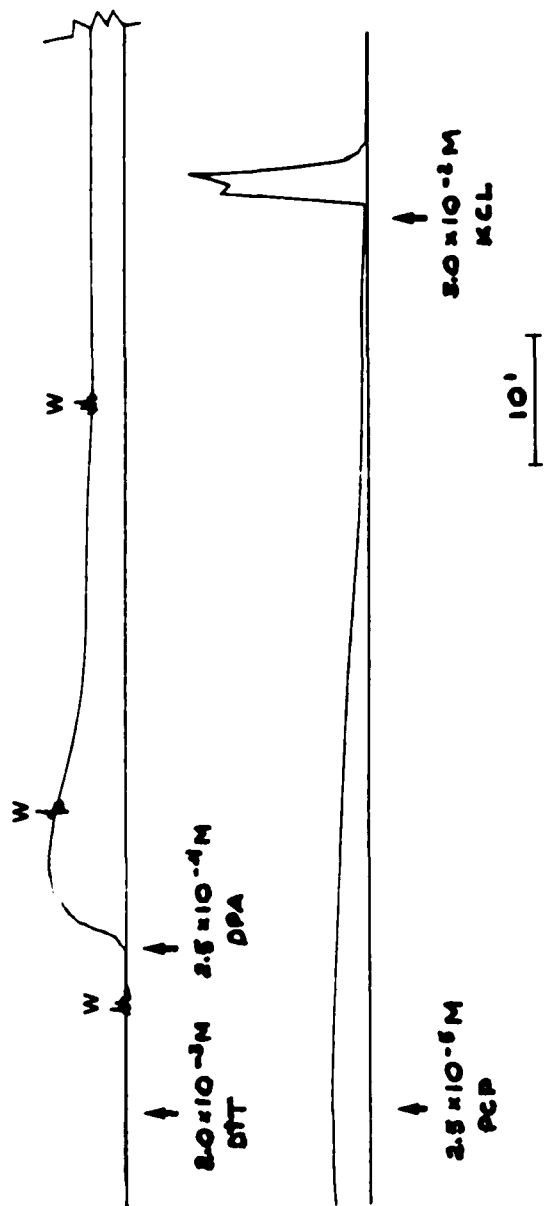


Table 1. Concentration dependence of PCP for antagonism of the sustained DPA response.

| PCP   | $T_{1/2}$ for DPA inhibition (min.)* | N |
|-------|--------------------------------------|---|
| 5uM   | $45.0 \pm 9.9$                       | 4 |
| 10uM  | $28.8 \pm 4.9$                       | 4 |
| 25uM  | $18.5 \pm 2.8$                       | 4 |
| 100uM | $7.5 \pm 7.0$                        | 3 |

\* Duration of time measured to obtain 50% inhibition of the sustained DPA contracture resulting from alkylation of frog rectus abdominis muscle. DPA alkylation was carried out by reduction with 2mM DTT for 20 minutes followed by washout and treatment with 0.25mM DPA for 20 minutes.

tracture following PCP washout was not observed.

In common with the channel blocking compounds, dTC (0.1mM; fig. 9) and  $\alpha$ -BTX ( $3 \times 10^{-7}$  M, fig. 10) were found to antagonize the sustained DPA contracture. Similar to PCP, once reversal was achieved with dTC, attempts at restoration of tension by repeated washout of the dTC proved to be unsuccessful.

#### 6. Effect of PCP and adiphenine on carbachol induced muscular contraction.

##### A. Effect on cumulative dose response to carbachol.

PCP is a potent inhibitor of carbachol induced muscle tension in the rectus abdominis muscle preparation (fig. 11). Cumulative log dose-response curves to carbachol in the presence of low doses, i.e., 1 $\mu$ M PCP, results in an increase in  $EC_{50}$  and decrease in Hill coefficient ( $P < .05$ ) as compared with control (from 1.87 $\mu$ M to 3.05 $\mu$ M and 1.92 to 1.34; respectively). However, there is no significant reduction in the maximal carbachol response obtained in the presence of this PCP dose (96 $\pm$ 2.0% of control). At higher doses of PCP, i.e., 5 $\mu$ M, a 65.4 $\pm$ 4% reduction in the maximum response ( $p < .01$ ) is observed, with no further significant effect on the  $EC_{50}$  or on Hill coefficient (1.72 $\mu$ M and 1.58, respectively).

Adiphenine inhibition of carbachol induced tension was also studied to serve as a reference compound for an agent established to act as an ion channel inhibitor. Like PCP, adiphenine proved to be a non-competitive inhibitor for the carbachol response. A maximal carbachol response of 76.4 $\pm$ 2.3% and 46.1 $\pm$ 5.2% of control tension was obtained in the presence of 5 $\mu$ M and 10 $\mu$ M concentrations, respectively (fig. 12). As was also the case with PCP, the lower concentration which resulted in a

Figure 9. Blockade of the sustained DPA contracture of rectus abdominis muscle by  $\alpha$ -BTX. The tension tracings of two halves of a single rectus abdominis muscle are shown in panel A (contd. in A<sup>1</sup>) and panel B (contd. in B<sup>1</sup>). Response to a test dose of carbachol (15 $\mu$ M) is followed by DTT reduction (2 mM-20 min) and DPA alkylation (0.5mM-20 min). The treatments of the two muscles are identical with the exception that  $2 \times 10^{-7}$  M BTX is administered to the muscle bath in panel B<sup>1</sup> as indicated.

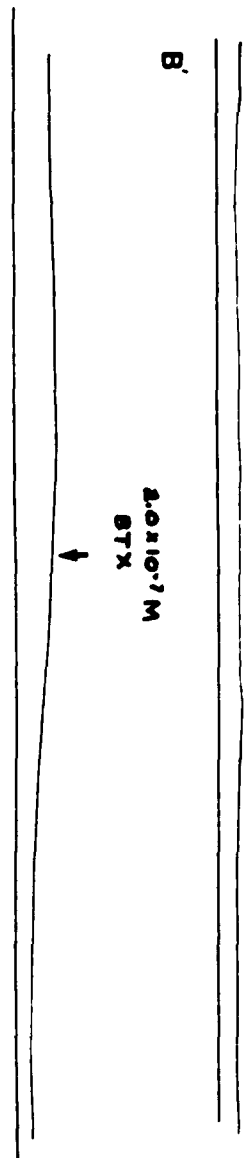
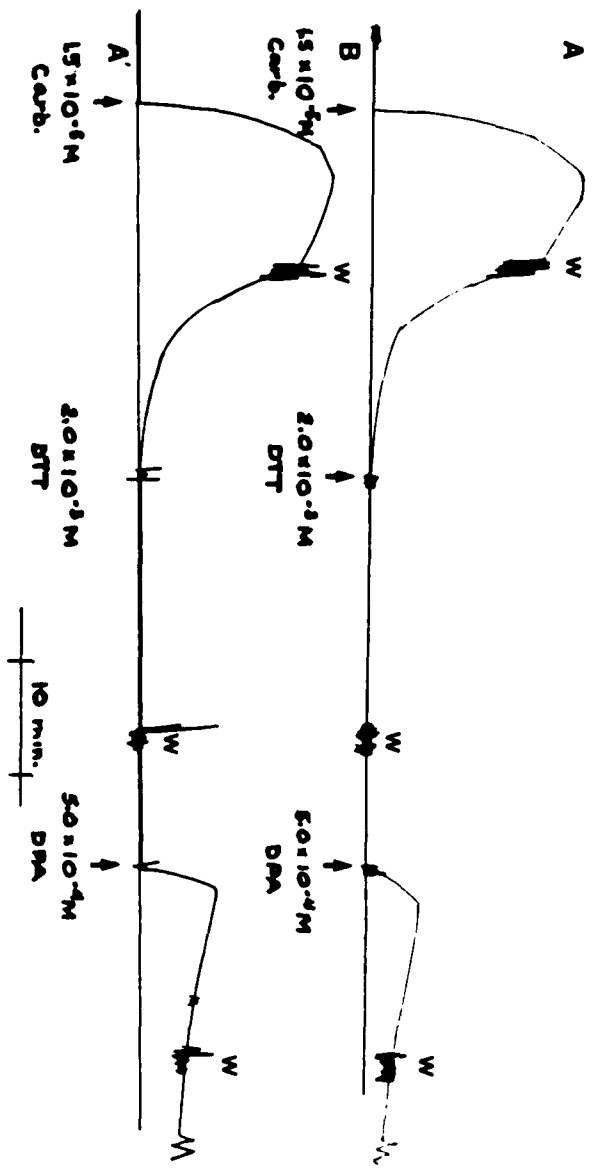


Figure 10. Blockade of the sustained DPA contracture of rectus abdominis muscle by dTC. The tracing shows the tension response of a muscle which has been reduced with DTT (2mM-20 min) and alkylated with 0.25 mM DPA for 20 min. The lasting response obtained following DPA washout is gradually blocked by addition of 0.1 mM dTC.

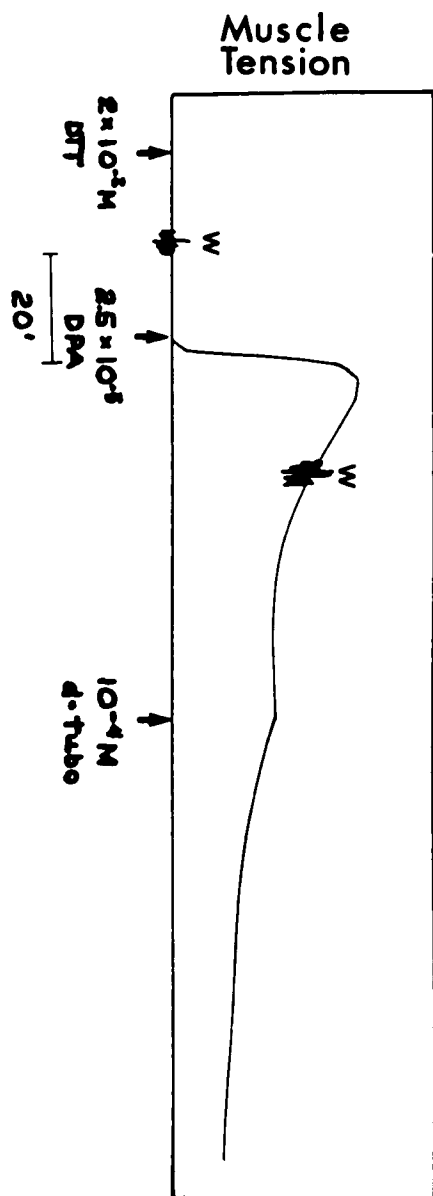


Figure 11. Effect of PCP on cumulative carbachol dose response curve.

Cumulative carbachol dose-response curves were generated by measuring muscle tension in response to the summation of carbachol doses added at 4 min intervals. The curves shown are for carb in the absence (---), or presence of 1 $\mu$ M (---) and 5 $\mu$ M (---) PCP. Each point represents the mean of at least 3 individual muscle preparations. The maximum carbachol response obtained in the presence of 1 and 5  $\mu$ M PCP is  $96.0 \pm 2.0\%$  and  $34.6 \pm 4.47\%$  of control, respectively. PCP at 1 $\mu$ M significantly alters the  $EC_{50}$  for carbachol ( $P < .05$ ) from 1.87 $\mu$ M in control (Hill coefficient = 1.92) to 3.05 $\mu$ M; but no significant change is observed (other than the reduction in maximal response) in the presence of 5 $\mu$ M PCP ( $EC_{50}$ =1.72 M and Hill coefficient=1.58).

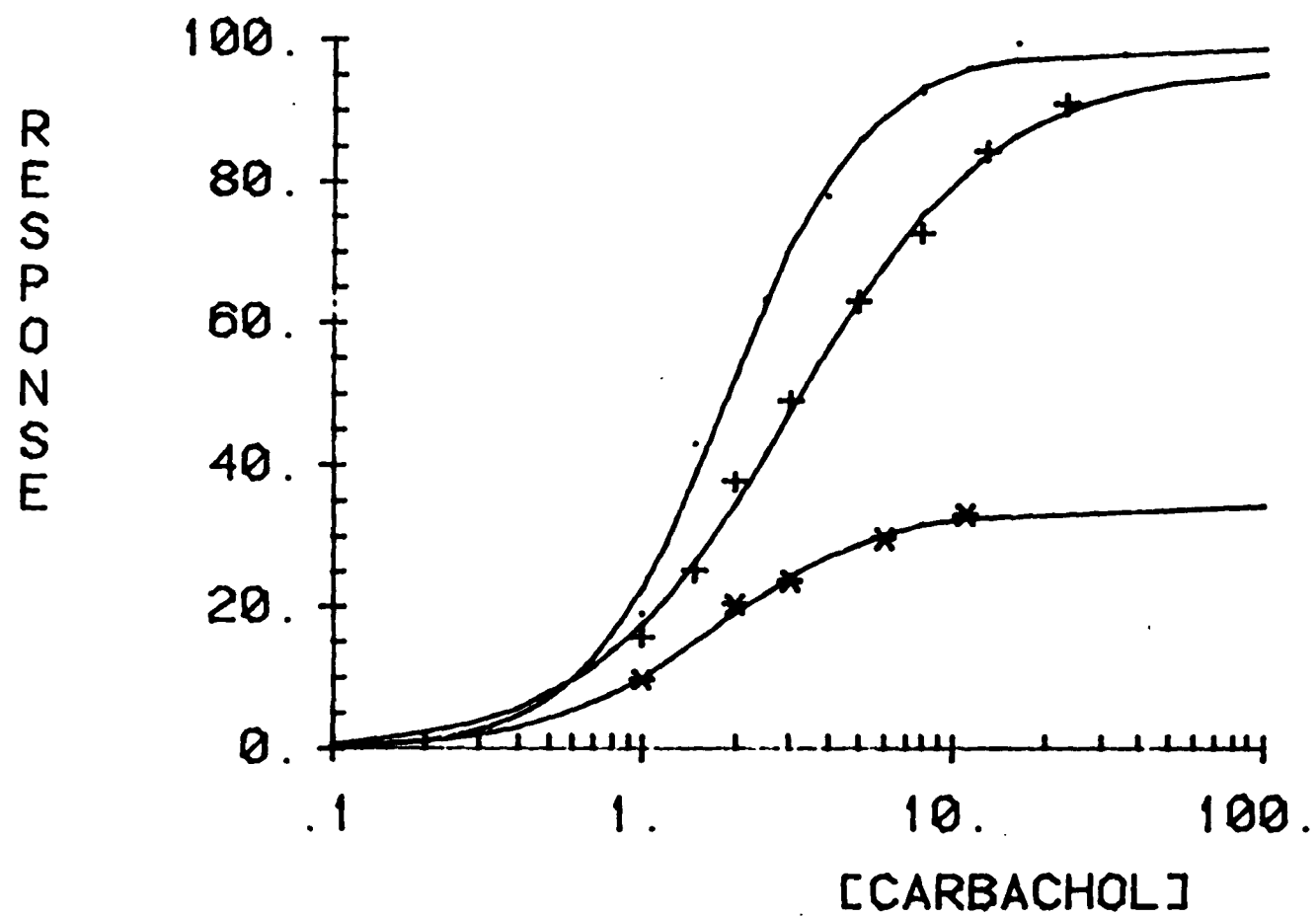
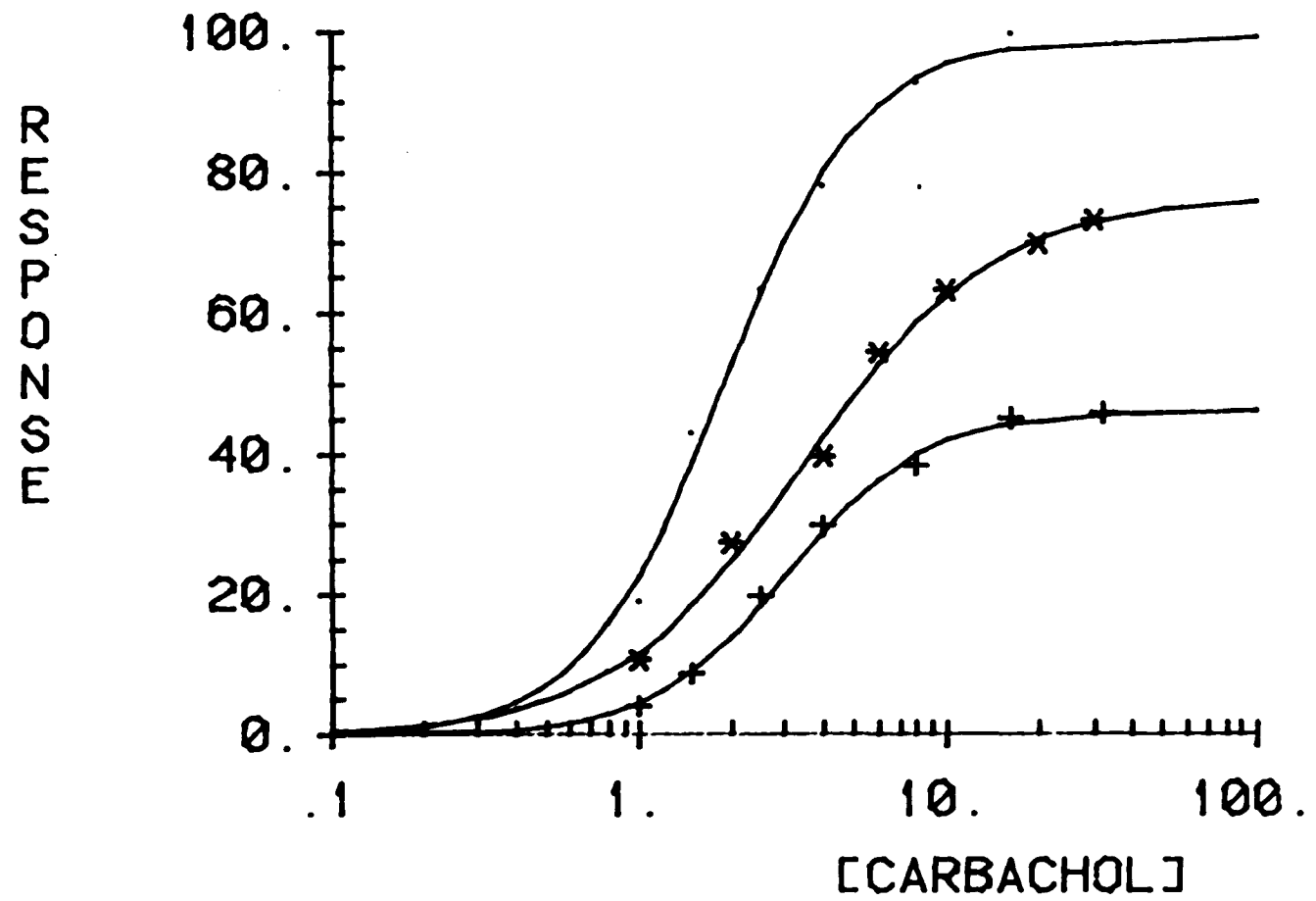


**Figure 12:** Effect of adiphenine on cumulative carbachol dose response curves. Cumulative carbachol dose-response curves were generated by measuring muscle tension in response to the summation of carbachol doses added at 4 min. intervals. The curves shown are for carbachol in the absence (- . -) or presence of 5 $\mu$ M (- \* -) or 10 $\mu$ M (- + -) adiphenine. Each point represents the mean of at least 3 individual muscle preparations. A significant reduction of the maximum response is obtained ( $P < .01$ ) with 76.4 $\pm$ 2.3% and 46.1 $\pm$ 5.2% of control tension in the presence of 5 $\mu$ M and 10 $\mu$ M adiphenine, respectively. The  $EC_{50}$  values determined are significantly increased to 3.01 $\mu$ M (Hill coefficient=1.39) with 5 $\mu$ M adip. ( $P < 0.05$ ) and 4.49 $\mu$ M (Hill coefficient=1.96) with 10 $\mu$ M adip. ( $P < 0.01$ ) relative to control values ( $EC_{50}$ =1.87; Hill coefficient=1.92).



relatively small decrease in maximal response gave a significantly reduced Hill coefficient ( $H=1.39\pm 13$ ,  $p<.05$ ), while the higher dose which resulted in greater than 50% reduction of the maximal response had no effect on Hill coefficient ( $H=1.96\pm .09$ ). The  $EC_{50}$  for carbachol in the presence of  $5\mu M$  and  $10\mu M$  adiphenine was  $3.01\mu M$  and  $4.49\mu M$ , respectively, compared to  $1.8\mu M$  for control muscles.

Both PCP and adiphenine enhance the apparent rate of desensitization induced by prolonged exposure of the muscle to a high concentration of carbachol (fig. 13). Since these observations are made following a prolonged preincubation of the muscle with the PCP or adiphenine (2 hrs), the fading of response cannot be attributed to a slow establishment of an equilibrium with antagonist subsequent to addition of the agonist. Equilibrium of the muscle with PCP is complete within the 2 hrs. of preincubation as indicated by the time course of PCP antagonism of carbachol activation as shown in fig. 17.

#### B. Sequential carbachol dose response curves.

Evaluation of PCP as an antagonist of carbachol induced muscle tension gave somewhat different results when individual doses of agonist were spaced an hour apart from one another (sequential dose response curve). When the concentration of carbachol was varied cumulatively, PCP exhibited non-competitive antagonist activity. In the presence of  $5\mu M$  PCP the maximal response to carbachol was about 35% of control and the  $EC_{50}$  to carbachol was unchanged. In contrast, when agonist concentration was varied sequentially only a small (13%) and not significant reduction in the maximal response was obtained. However, a significant increase in carbachol  $EC_{50}$  was observed ( $4.49\mu M$ , see fig. 14). Untreated control muscles gave almost identical dose response

Figure 13. Decline in response to a prolonged high concentration of carbachol. Exposure of rectus muscle to 7.5 $\mu$ M carbachol ( 4xED<sub>50</sub>) for a sustained period of time results in a slow decline in tension to 80-90% (n=4) of the initial value (center panel). In muscle preincubated for 120 min with 5 $\mu$ M phencyclidine (upper panel) or 5 $\mu$ M adiphenine (lower panel) the decline in response to 7.5 $\mu$ M carbachol is rapid and much more pronounced than that of control muscle, suggesting an enhanced rate of desensitization.

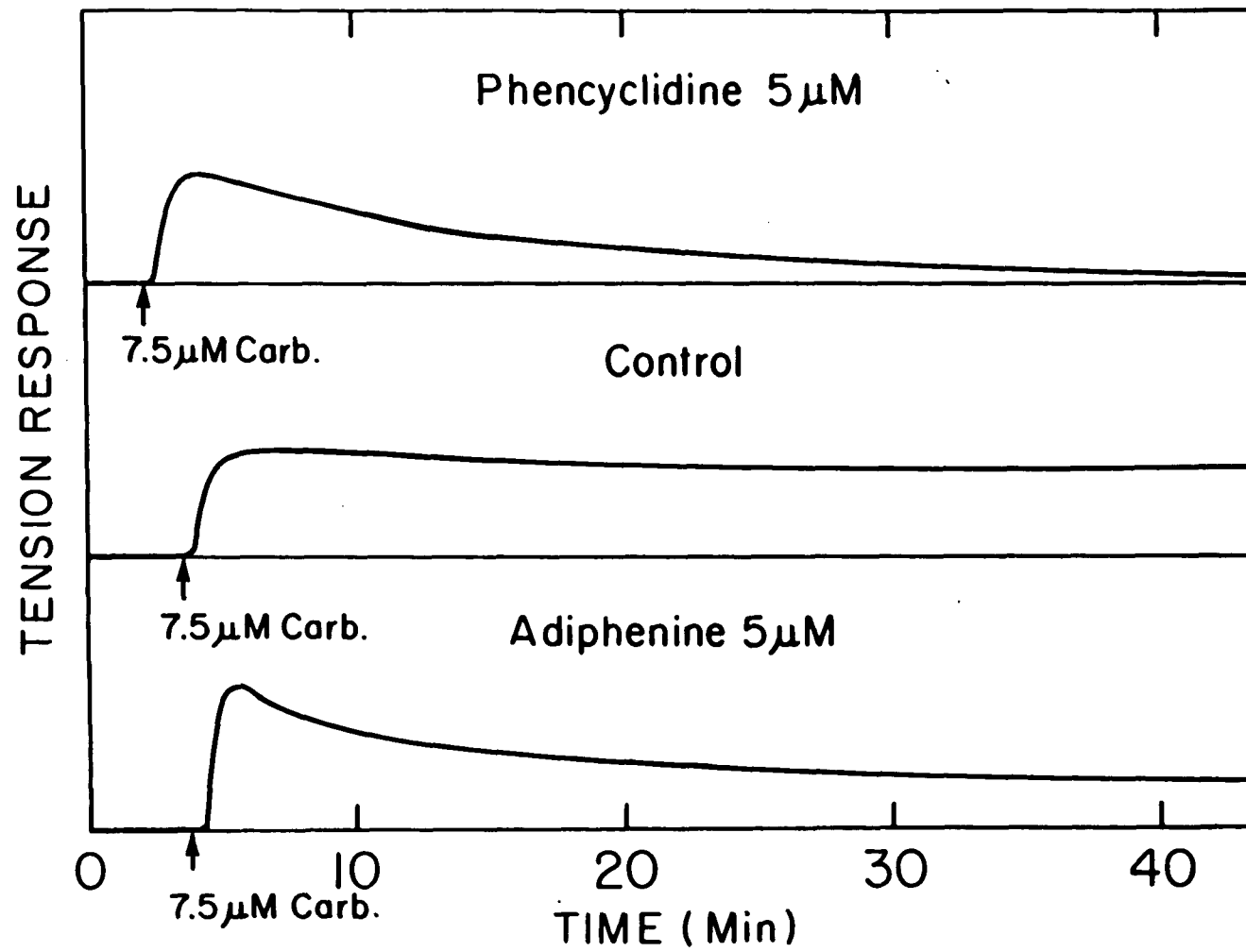
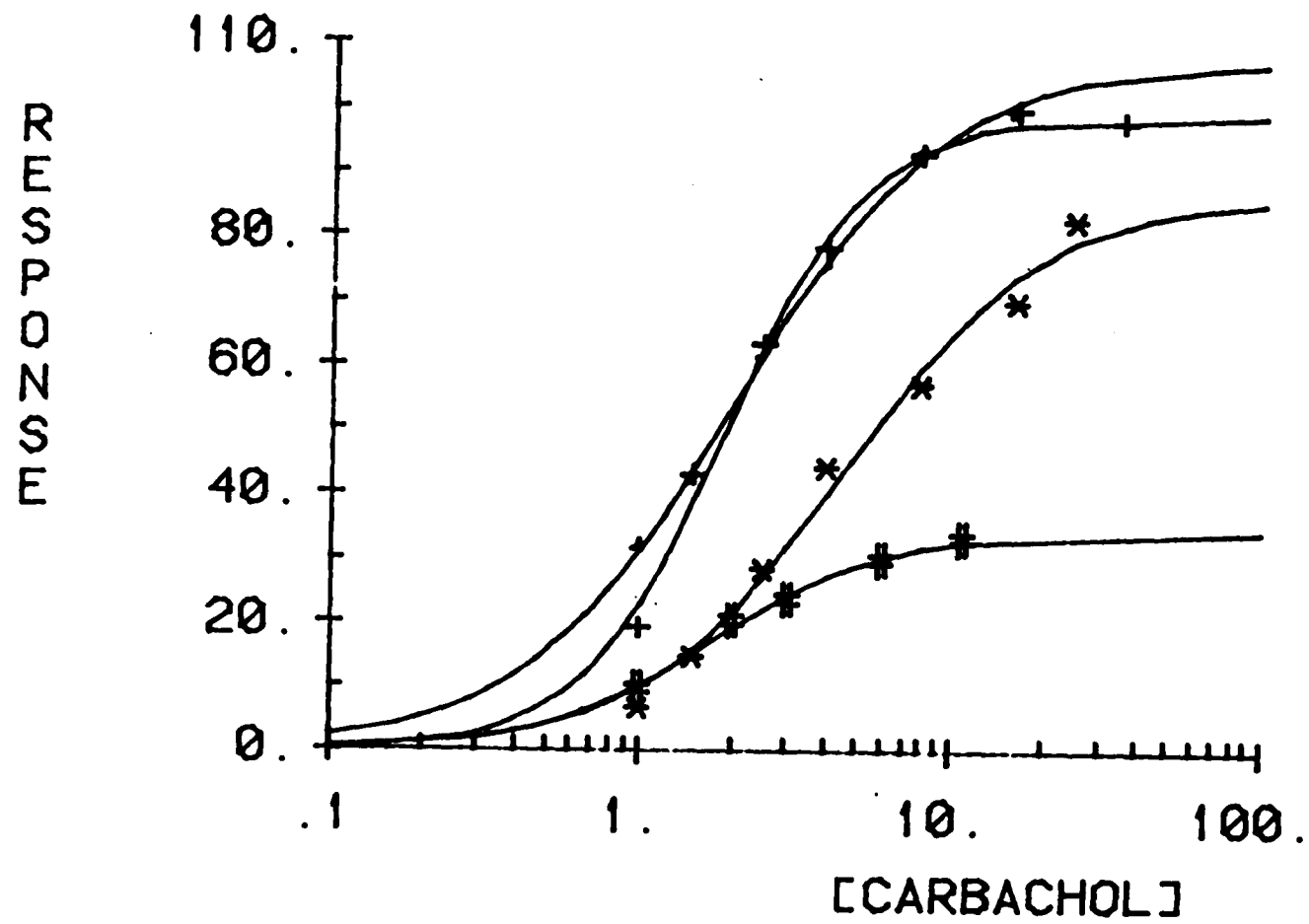


**Figure 14.** The effect of PCP on sequential and cumulative dose response curves to carbachol. Carbachol dose response curves that were generated by the sequential method (individual doses and washout spaced at one hr intervals) and by the cumulative method (summation of doses added at 4 min intervals) are shown. Dose-response curves obtained by each of these methods is shown for untreated muscles and muscles that were pretreated with 5 $\mu$ M PCP for 120 mins. All data points represent average values obtained for a minimum of 3 individual muscle preparations. No significant difference is observed between EC<sub>50</sub> values or Hill coefficients obtained for untreated muscle with cumulative (---) versus sequential (—) methods (EC<sub>50</sub>=1.87 $\mu$ M, Hill coefficient=1.92 and EC<sub>50</sub>=1.99 $\mu$ M, Hill coefficient=1.79, respectively). In the presence of 5 $\mu$ M PCP, significantly different results are obtained by the cumulative (-\*-) vs sequential (-#-) methods. The maximum response to carbachol was significantly reduced when agonist was varied cumulatively, but not when varied sequentially. PCP significantly increased the EC<sub>50</sub> value for the response to carbachol when the concentration of carbachol was varied sequentially but not when varied cumulatively.



curves for carbachol for the cumulative or sequential variations of agonist concentration. Fig. 14 shows the difference in the carbachol dose response in the presence of  $5\mu\text{M}$  PCP when done by cumulative or sequential methods.

With adiphenine, unlike PCP, the nature of blockade is not effected by the method used to construct the dose response curve, i.e., antagonism is not enhanced by the sustained presence of agonist which occurs with the cumulative technique (fig. 15). No significant difference was observed for the maximal response to carbachol, the  $\text{EC}_{50}$ , or the Hill coefficient when cumulative or sequential dose response curves were experimentally determined in the presence of  $10\mu\text{M}$  adiphenine.

#### 7. Effect of repetitive agonist doses on PCP and adiphenine inhibition of muscle contraction.

The basis for the insurmountable antagonist activity produced by PCP seen when the concentration of carbachol was varied cumulatively is suggested by the results of experiments described below. A fixed schedule of agonist addition and washout in the continued presence of PCP following an initial preincubation with PCP ( $5\mu\text{M}$ ) or adiphenine ( $5\mu\text{M}$ ) is shown in figure 16. Here the same dose of carbachol is given sequentially; the contact time is 7 minutes, the dose interval is 12 minutes. This contrasts to the dose interval of 60 min. used above. A consistent equal response is observed with each agonist addition in control and adiphenine equilibrated muscles. However, in the presence of PCP the responses to carbachol are seen to decrease with successive additions of agonist. This decline in agonist response in the presence of PCP is not complete; rather, it reaches a plateau at the fifth dose

Figure 15. The effect of adiphenine on sequential and cumulative dose response curves to carbachol. Carbachol dose response curves are shown which were generated by the sequential method (individual doses and washout spaced at one hour intervals) and by the cumulative method (summation of doses added at 4 min. intervals). Dose response curves obtained by each of these methods are shown for untreated muscle and muscle that had been pretreated with  $10\mu\text{M}$  adiphenine for 120 mins. All data points represent average values obtained from a minimum of 3 individual muscle preparations. No significant difference is observed between the  $\text{EC}_{50}$  values for carbachol or the Hill coefficients obtained for untreated muscle when the dose response curves were constructed by either the cumulative (---) or the sequential (-#-) method ( $\text{EC}_{50}=1.87\mu\text{M}$ , Hill coefficient=1.92 and  $\text{EC}_{50}=1.99\mu\text{M}$ , Hill coefficient=1.79, respectively). In contrast to PCP, the antagonism produced by adiphenine was the same regardless of how the concentration of agonist was varied. Adiphenine behaved as a simple non-competitive antagonist in each case, reducing only the maximal response to carbachol ( $46.1\pm 5.2\%$  and  $51.0\pm 6.1\%$  for sequential and cumulative respectively) and not significantly altering the  $\text{EC}_{50}$  or the Hill coefficient.

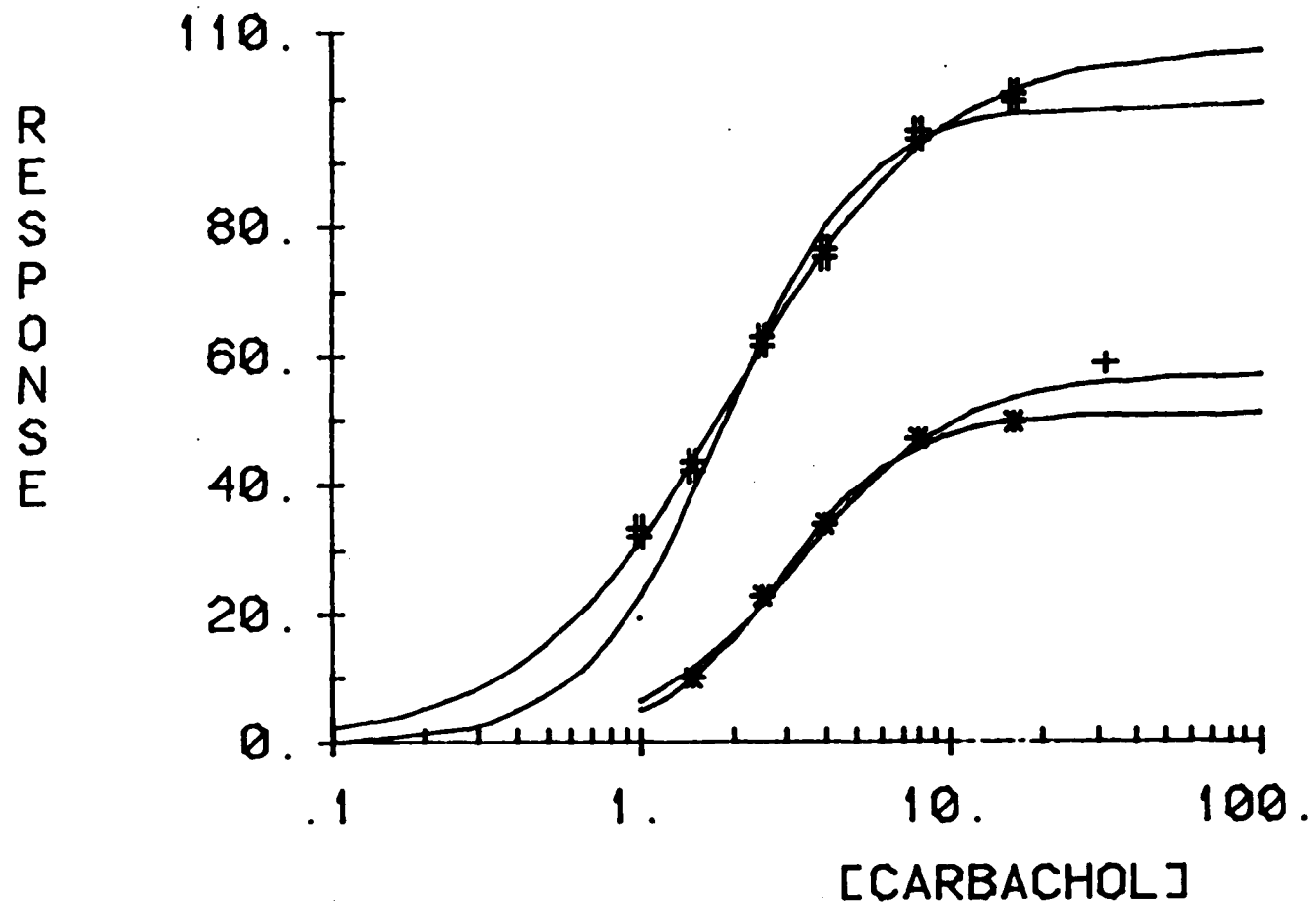
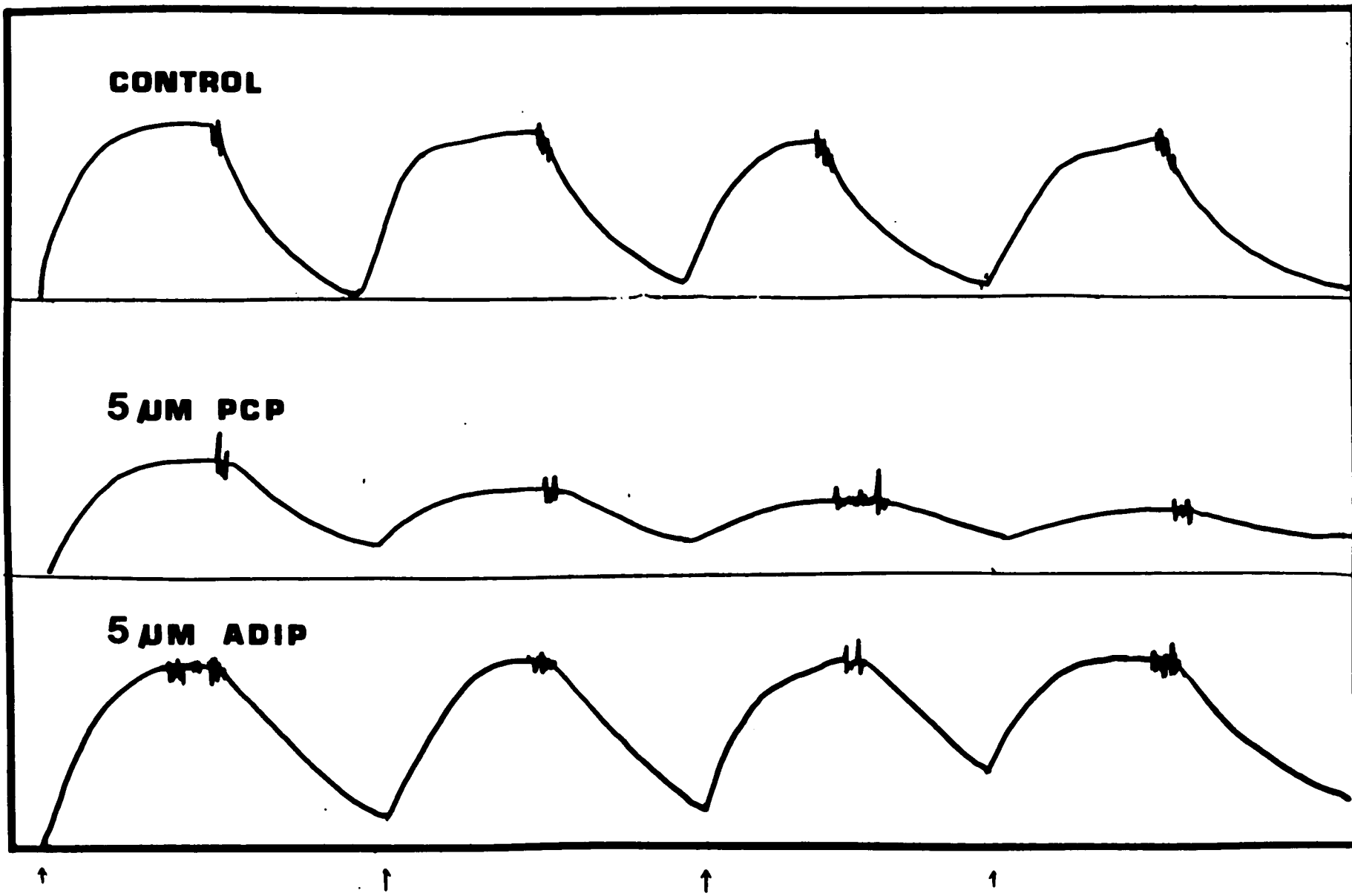


Figure 16. Fixed schedule carbachol responses in frog rectus abdominis preparation — Effect of PCP and adiphenine. Addition of  $7.5\mu\text{M}$  carbachol is indicated by the arrows. After 7 min of agonist exposure, the muscles are washed and allowed to relax for 5 min after which time the cycle is repeated. Tracings show the effect of 4 repeated cycles. The upper panel is the control muscle while the middle and lower panel are muscles that have been equilibrated for 2 hrs with  $5\mu\text{M}$  PCP or  $10\mu\text{M}$  adiphenine, respectively. Control tissues show consistent responses to the sequential addition of carbachol on this dose schedule. A decrementing response to carbachol is observed with PCP but not with adiphenine treatment.

TENSION

**CONTROL****5  $\mu$ M PCP****5  $\mu$ M ADIP**

(on this dose time schedule) which is  $33.9\% \pm 3.3$  of the first response. After reaching this plateau, a 60 min. period without an agonist dose, but in the continual presence of PCP, is accompanied by a restoration of the response to the initial level ( $89.3 \pm 6.4\%$  ( $n=3$ )). The results obtained indicate that the presence of agonist causes development of an additional level of blockade by PCP which returns gradually to the original equilibrium level in the absence of agonist activation. That a two hour preincubation was sufficient to fully obtain equilibrium with PCP is indicated by the results of separate experiments on the time course for development of the PCP blockade (fig. 17). Adiphenine did not behave in this manner.

#### 8. Effect of PCP concentration on agonist enhanced PCP inhibition of muscle response.

The original equilibrium inhibitory response produced by PCP is perturbed by the prolonged or frequent presence of an agonist; this results in development of a new equilibrium state such that PCP becomes a more potent antagonist. The response obtained with each successive agonist challenge on a fixed time-dose schedule was determined in the presence of various concentrations of PCP. Analysis of the tension developed as a function of agonist dose number (in the sequence administered) indicates a PCP concentration dependence for both the initial response to agonist and the approach to a new equilibrium with a greater degree of block (see fig. 18). Evaluation of the dose dependency for inhibition by PCP was performed for both the first agonist dose, which approximates to the original equilibrium with PCP in the absence of agonist activation, and the fifth response, which approximates the new equilibrium of PCP with agonist activated receptors (fig.

Figure 17. Time course for development of the antinicotinic action of PCP in rectus abdominis muscle. Muscles preincubated with  $5\mu\text{M}$  PCP are challenged with a test dose of  $7.5\mu\text{M}$  carbachol ( $4\times\text{EC}_{50}$ ) at the indicated times. The time of PCP preincubation is plotted against the % of control (untreated muscle) response to the test dose of carbachol. Each point represents the mean of four different muscle preparations ( $\pm\text{S.E.M.}$ ). The maximal inhibition by this concentration of PCP was obtained by 2 hours indicating complete equilibration of the tissue with the drug in this time period.

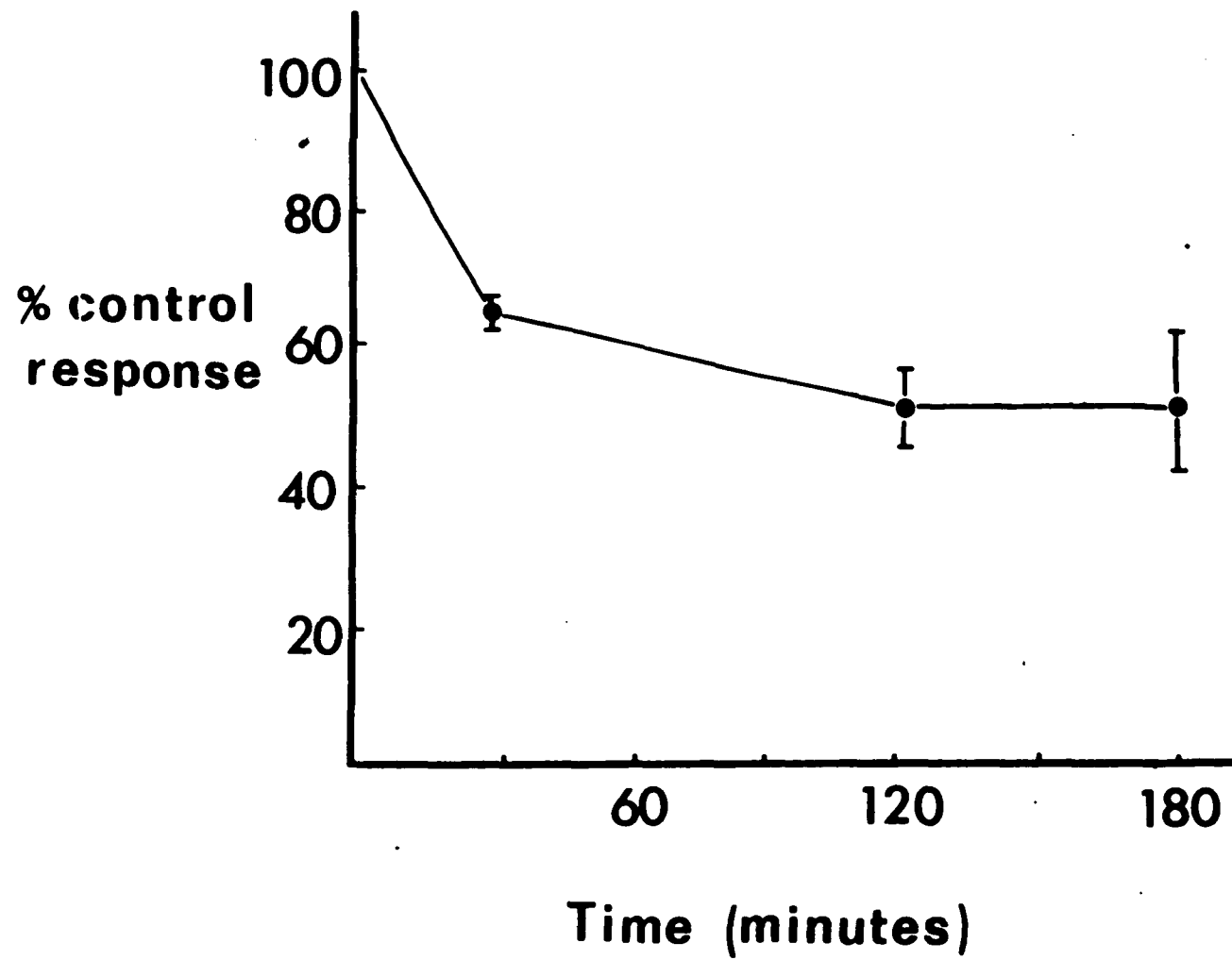
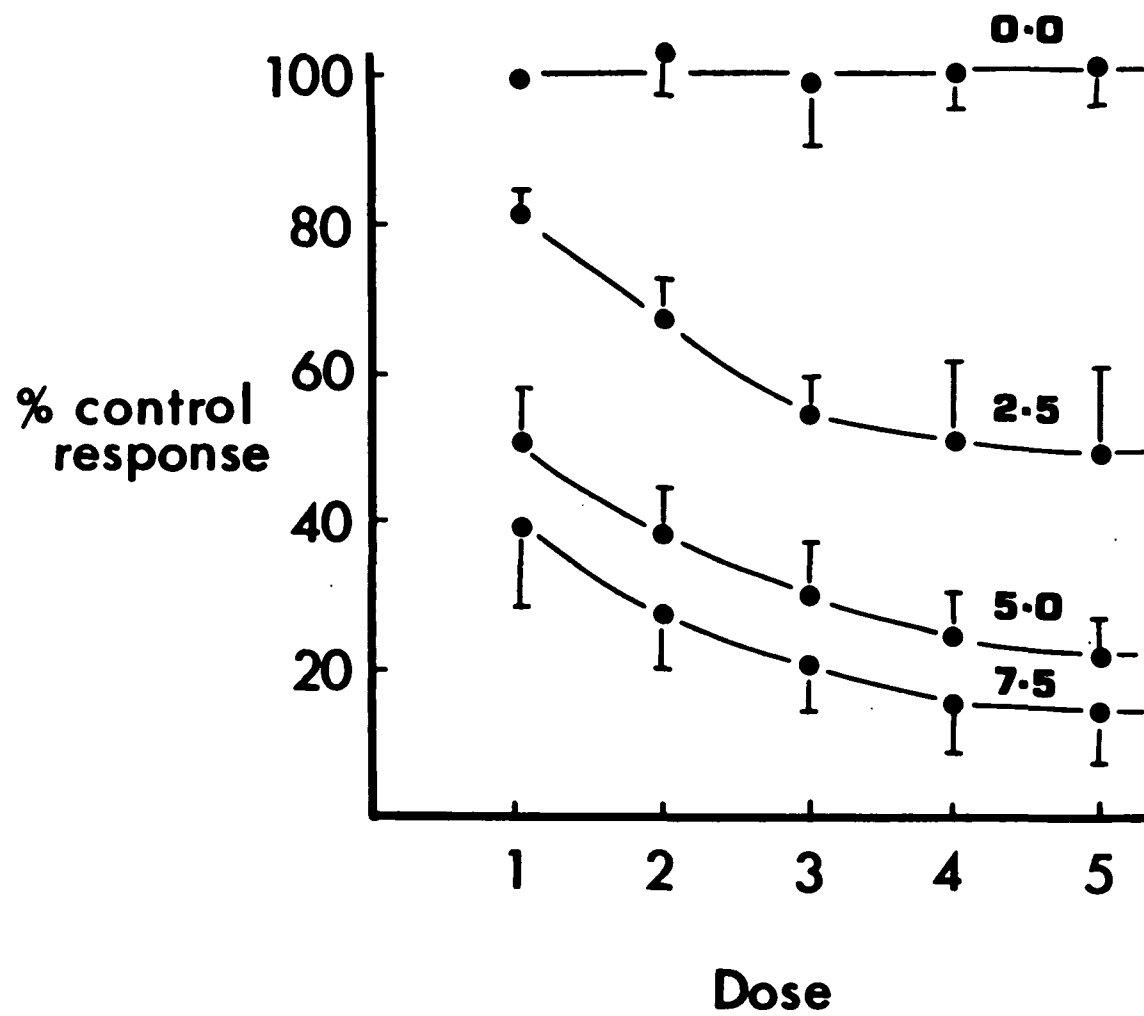


Figure 18. Effect of PCP concentration on response to fixed schedule carbachol activation. Muscles are preincubated with PCP for 2 hrs prior to testing. A total of 5 test doses of carbachol ( $7.5\mu\text{M}$ ) are used, each having a 12 min cycle (7 min in the presence of agonist followed by a 5 min washout). The percent of control response to  $7.5\mu\text{M}$  carbachol in the absence of PCP treatment is plotted as a function of agonist dose number for each PCP concentration employed. All points represent the mean of results obtained for 4 different muscle preparations ( $\pm\text{S.E.M.}$ ). The  $\text{EC}_{50}$  for PCP as an antagonist depends on previous agonist exposure of the muscle. Both the initial carbachol response and the fifth carbachol response are inhibited by PCP in a dose-dependent manner.



19). The  $IC_{50}$  values of PCP for the two responses were significantly different ( $P < .05$ ,  $5.8 \pm 0.5 \mu M$  and  $2.4 \pm 0.8 \mu M$  for the first and fifth responses, respectively). These data suggest that open channels (after agonist receptor activation) have at least a 2-fold higher affinity for PCP than do closed channels, assuming that PCP blockade in both cases is due to binding to a channel site.

9. Effect of agonist concentration on enhancement of PCP inhibition.

The rate of enhancement of PCP blockade following a fixed schedule agonist treatment is independent of the concentration of agonist used for activation (fig. 20). Using the 5 dose-12 min. cycle of agonist doses, 2 and  $7.5 \mu M$  carbachol caused no desensitization of control muscle but  $15 \mu M$  caused a 30% desensitization. However on muscles equilibrated with PCP the development of enhanced PCP blockade was the same for all 3 doses of carbachol.

10. Effect of elevated  $Ca^{++}$  concentration on agonist enhanced PCP inhibition.

Since agonist induced desensitization of nicotinic receptors is  $Ca^{++}$  dependent (Manthey, 1966), the effect of differing  $[Ca]$  on PCP was investigated. Elevation of the  $Ca^{++}$  concentration from  $2.0 mM$  to  $6.6 mM$  results in a significant desensitization with repetitive administration of  $7.5 \mu M$  carbachol in control muscle ( $23.6 \pm 8.2\%$  inhibition in response for the fifth agonist dose of the series, fig. 21). However in the presence of  $5 \mu M$  PCP no enhancement in the rate of decline of the carbachol response was observed when high  $Ca^{++}$  was compared to normal  $Ca^{++}$  concentration. The percentage of control tension obtained after the fifth agonist dose was  $22.6 \pm 5.1\%$  and  $24.4 \pm 6.2\%$  for  $2.0$  and  $6.6 mM$  calcium, respectively. Thus the enhanced blockade by PCP following

Figure 19. PCP blocking action as a function of agonist exposure. Data from experiments similar to those shown in fig 17 are plotted to demonstrate the blockade by various doses of PCP as a function of the repeated agonist dose. All points represent the mean ( $\pm$ S.E.M.) of at least 3 different muscle preparations. The percentage of control responses obtained by the first dose of carbachol in the series (closed circles) and the response to the fifth dose (open circles) are shown. The  $IC_{50}$  of PCP for the initial response ( $5.8 \pm 0.5 \mu M$ ) is significantly greater ( $P < 0.05$ ) than the  $IC_{50}$  for the final response ( $2.4 \pm 0.8 \mu M$ ). The blockade for all levels of PCP dose reaches a maximum by the fifth carbachol dose on this schedule.

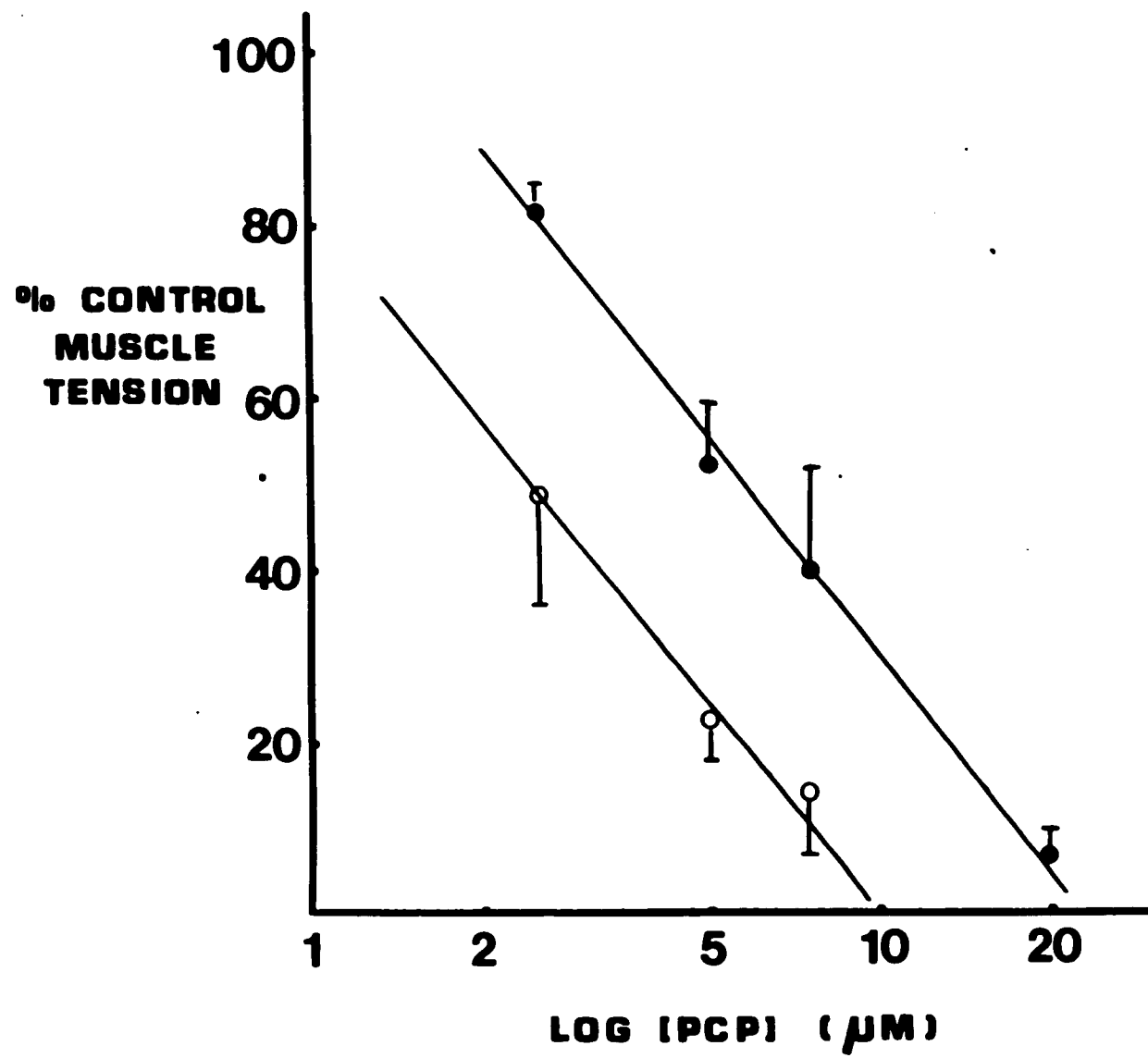


Figure 20. Muscle tension response to fixed schedule agonist treatment — Effect of agonist concentration. Muscles were either preincubated with 5 $\mu$ M PCP for two hrs (open symbols) or were without pretreatment (solid symbols). Fixed schedule agonist stimulation (12 min cycle — 7 min exposure followed by 5 min washout) utilizing 2.0 $\mu$ M (triangles), 7.5 $\mu$ M (circles) or 15.0 $\mu$ M (squares) carbachol is carried out as described in fig 16. The percentage of response is plotted as a function of the dose number in the series with the response to the initial agonist dose set at 100%. Each point represents the mean of values obtained by at least three muscle preparations ( $\pm$ S.E.M.). Repetitive application of 15.0 $\mu$ M carbachol results in some desensitization of control muscles but 2.0 and 7.5 $\mu$ M do not desensitize. The rate and degree of tension decline with repetitive administration in the presence of PCP is the same for all three dose levels of carbachol.

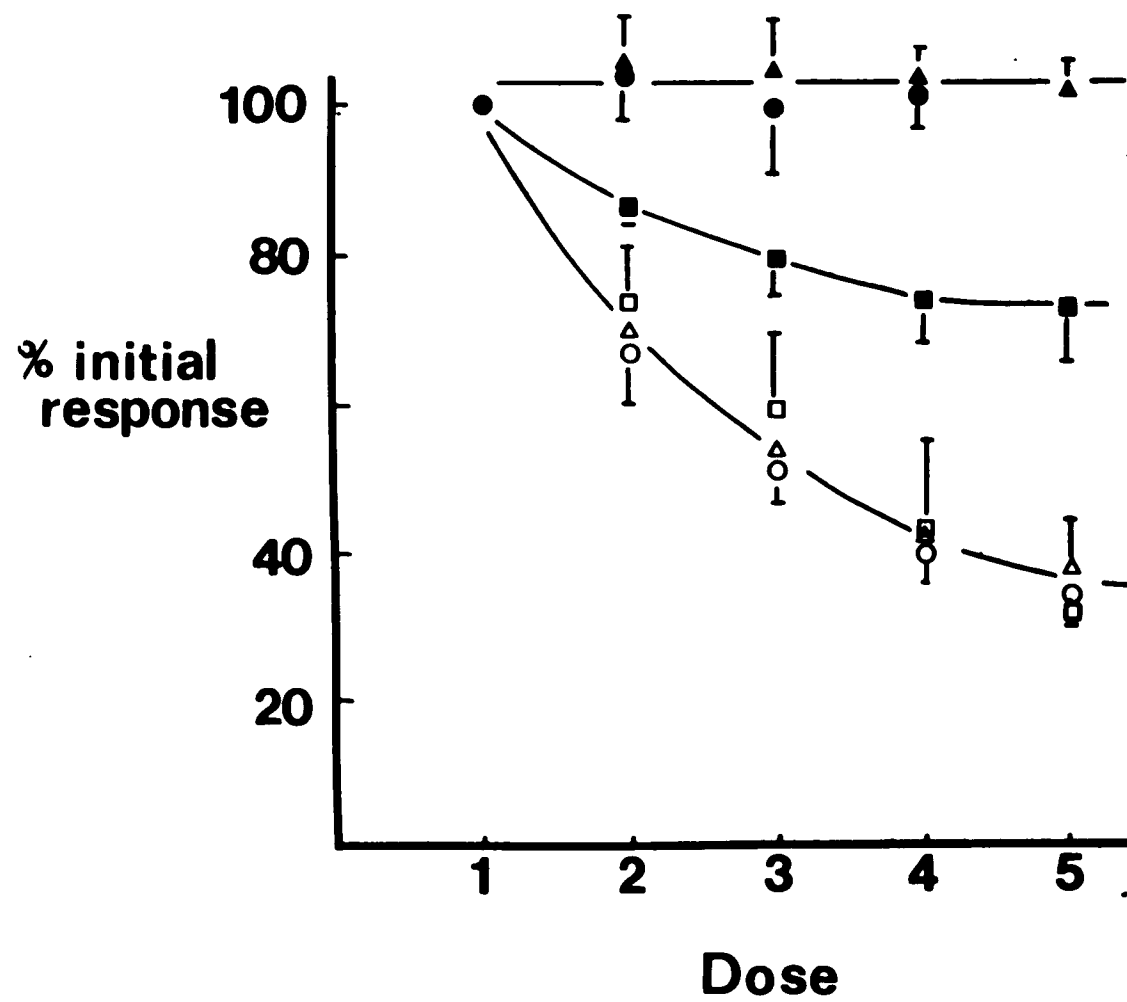
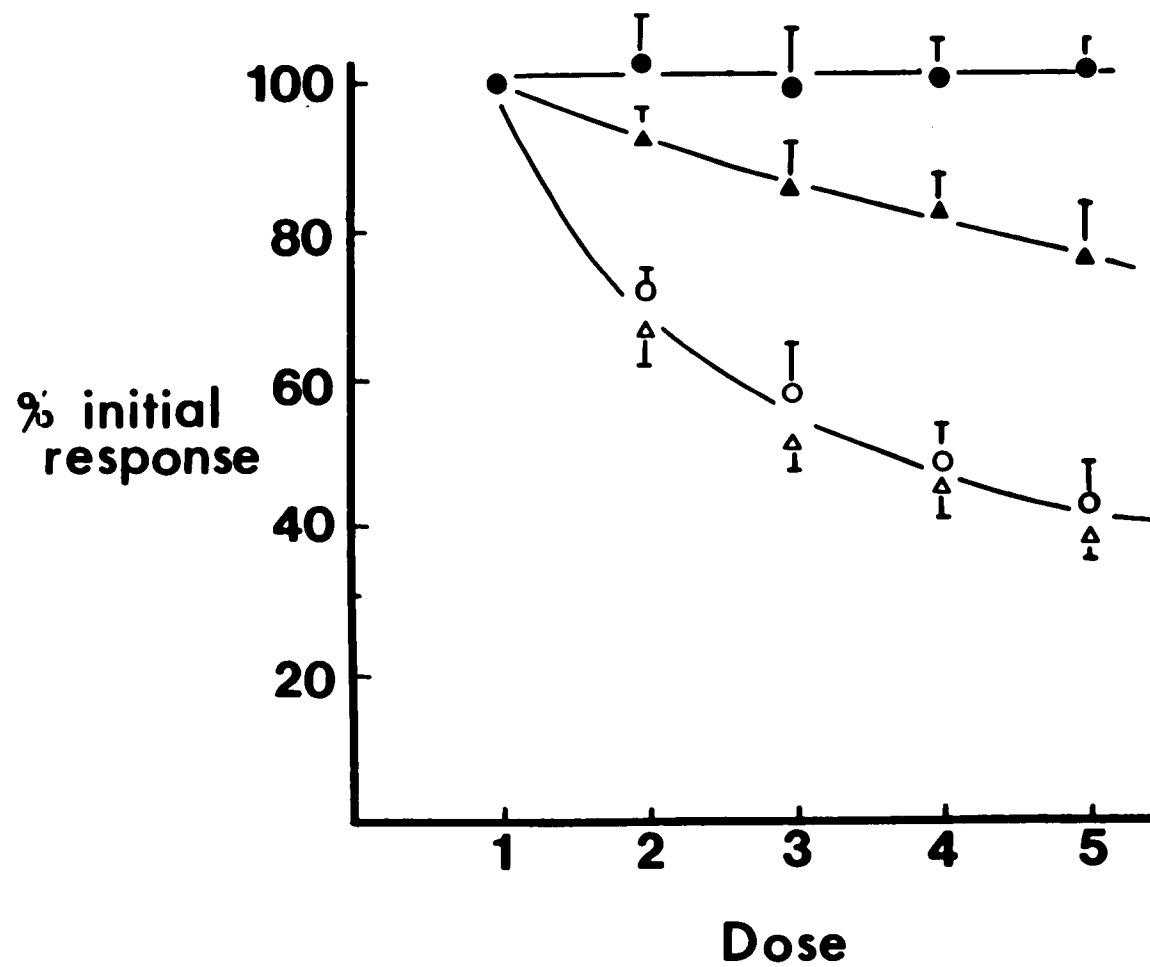


Figure 21. Tension development in response to fixed schedule agonist treatment — Effect of elevated  $\text{Ca}^{++}$  concentration. Muscles were either preincubated with  $5\mu\text{M}$  PCP for 2 hrs (open symbols) or untreated (solid symbols). Fixed schedule agonist stimulation utilizing  $7.5\mu\text{M}$  carbachol was carried out as described in fig. 16. The amphibian Ringer's solution bathing the muscles throughout the experiment contained either normal  $2.0\text{mM}$   $\text{Ca}^{++}$  (circles) or high  $6.6\text{mM}$   $\text{Ca}^{++}$  (triangles). The percent of control response obtained by the first agonist dose is plotted as a function of the dose number in the series. Each point represents the mean values obtained by at least three different muscle preparations ( $\pm\text{S.E.M.}$ ). Desensitization by  $7.5\mu\text{M}$  carbachol alone is observed in control muscle bathed in high  $\text{Ca}^{++}$  Ringer's. However no effect of high  $\text{Ca}^{++}$  is observed on the rate or extent of decline in tension resulting from repetitive agonist administration in those muscles preequilibrated with  $5\mu\text{M}$  PCP.



agonist activation of receptors does not appear to be due to summation with additional agonist dependent desensitization caused by increased Ca entry over this range of Ca concentration.

11. Radioligand binding experiments - Intact muscle. [ $^{125}$ I]  $\alpha$ -BTX labelling time period.

Since the physiological experiments were performed using the frog rectus abdominis muscle, it would have been most desirable to use this same muscle for quantitative [ $^{125}$ I]  $\alpha$ -BTX binding experiments. However, due to the prolonged period of time necessary for diffusion of toxin to all fibers to give complete labelling of the receptors with toxin (3-7 days at 4°C with 5nM  $\alpha$ -BTX) and the potential problem of degradation of receptors (proteolysis) during incubation of muscle for such a long period, the binding studies were done on another muscle. The frog sartorius muscle which is composed of a thin sheet of fibers was selected. Toxin binding to this muscle was previously reported (Katz and Miledi, 1971).

The time course for labelling of intact sartorius muscle incubated in  $1 \times 10^{-8}$  M [ $^{125}$ I]  $\alpha$ -BTX in amphibian Ringer's solution at 4°C and followed by extensive washing is shown in figure 22. The maximal  $\alpha$ -BTX bound per muscle ( $197 \pm 15$  fmoles) occurred in about 48 hrs of incubation. The difference in amount of toxin bound at 24, 48 and 96 hrs is not significant. In all experiments reported here involving [ $^{125}$ I]  $\alpha$ -BTX binding to intact sartorius muscle, toxin is incubated with muscle for 40 hrs at 4°C.

During the post mortem period of incubation with toxin there is no detectable loss in the number of  $\alpha$ -BTX binding sites per muscle (see table 2). This is indicated by the same binding of toxin when muscles

Figure 22. Time course of [ $^{125}$ I]  $\alpha$ -BTX binding to frog sartorius muscle. Muscles were incubated in groups of 4 in 1 ml of amphibian Ringer's solution containing 10nM [ $^{125}$ I]  $\alpha$ -BTX at 4° C. At various times muscles were removed, rapidly washed free of toxin (< 5 min) with five changes of 20 ml of iced amphibian Ringer's solution and blotted dry. Individual muscles were minced and homogenized in 0.5 ml of cold 2% Triton X-100 and allowed to extract overnight at 4° C. An aliquot of the extract was subjected to the Con A assay for receptor-bound [ $^{125}$ I]  $\alpha$ -BTX. Results are the average ( $\pm$ S.E.M.) of 4 individual muscles.

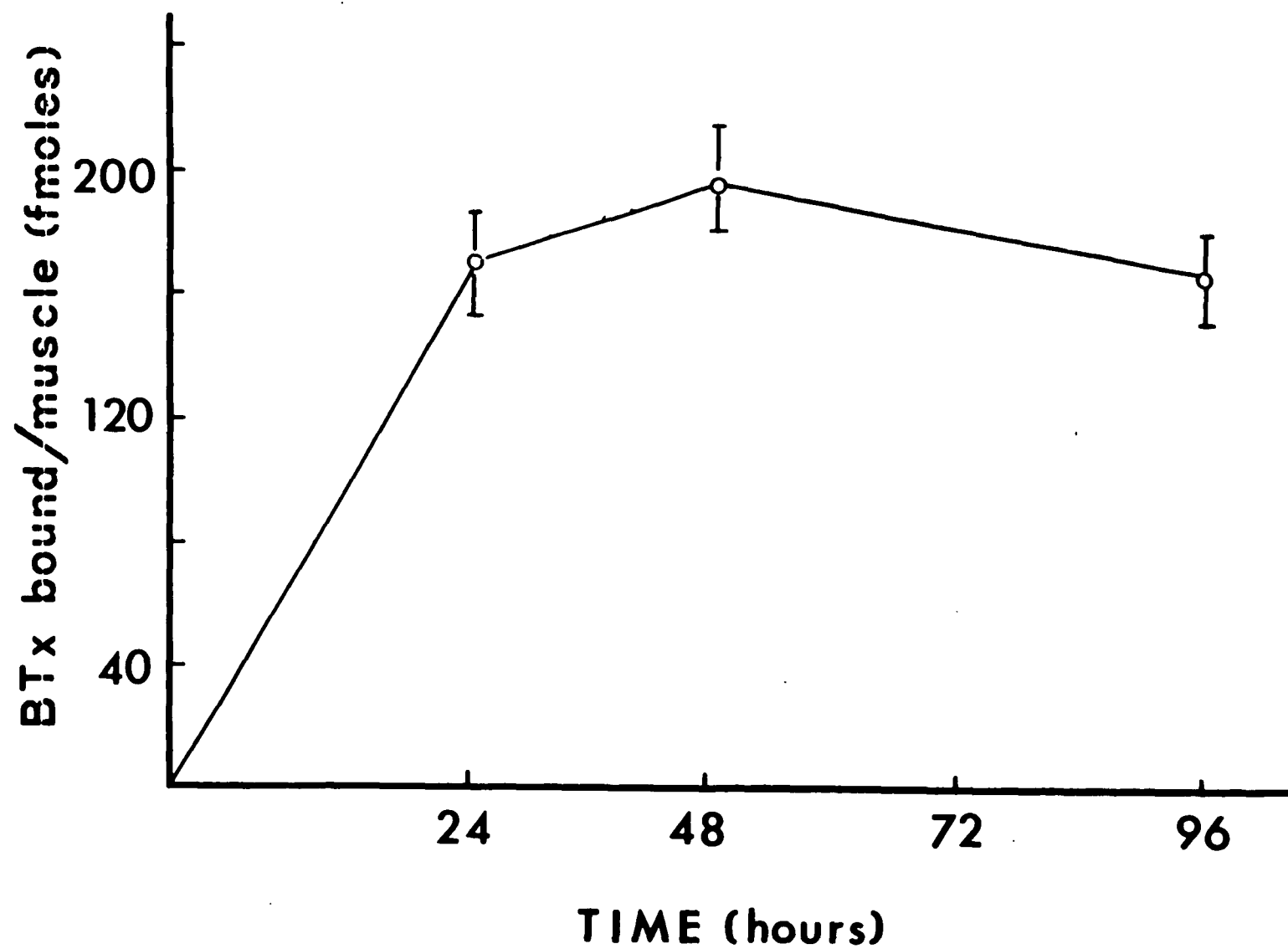


Table 2. Post mortem stability of AChR sites on whole sartorius muscle kept at 4°C. \*

| Preincubation:                           | Con A assay<br>(fmol bound/muscle) | n |
|------------------------------------------|------------------------------------|---|
| a) none                                  | 175.5 ± 30.5                       | 4 |
| b) amphibian Ringer's<br>72 hrs. at 4°C. | 170.4 ± 32.4                       | 4 |

\*Muscles were either assayed immediately (a) or preincubated for 72 hours at 4°C. in 2ml of amphibian Ringer's solution (b) and then assayed. Each muscle was minced and individually homogenized in 0.5ml 2% Triton X-100 and extracted for 1 hour at 4°C. The supernatants were removed by centrifugation for 1 minute in a Beckman microfuge followed by labelling with  $1 \times 10^{-8}$  M [ $^{125}$ I]  $\alpha$ -BTX for 24 hours at 4°C. An aliquot of the labelled Triton extract was assessed for  $\alpha$ -BTX binding using the Con A assay method.

were kept in amphibian Ringer's for 72 hrs at 4°C compared to detergent extraction of receptors from fresh muscle followed by toxin labelling of the solubilized nAChR ( $170 \pm 32.4$  and  $175 \pm 30.5$ , respectively). It is concluded from this finding that toxin binding sites on the muscle are stable during the usual duration of toxin labelling (40 hrs.).

#### 12. nAChR extraction time course.

A study of the time course for extraction of prelabelled  $\alpha$ -BTX binding sites in intact muscle by 2% Triton X-100 indicates a rapid solubilization of receptor from homogenized sartorius muscle (fig. 23). Within 1 hr, the first time point shown in fig. 23, extraction is already complete. No loss of toxin binding material is observed in the soluble extract during 24 hrs, indicating stability of the labelled receptors in detergent solution. Extraction of whole muscle (not homogenized) by incubation in 2% Triton X-100, by comparison with homogenized muscle, results in a relatively slow time course for extraction of  $\alpha$ -BTX binding material. However, by 73.5 hrs 100% of the toxin binding material is extracted from the intact muscles. This finding suggests not only that the solubilized receptors are resistant to enzymatic degradation but also that there is no significant dissociation of toxin from the receptor during this prolonged time period. For all practical purposes,  $\alpha$ -BTX binding to nAChR can be considered irreversible under the conditions employed. In order to minimize the time required for receptor solubilization, all muscles were homogenized in 2% Triton X-100.

#### 13. Internal vs. external nAChR.

The proportions of externally directed and intracellular  $\alpha$ -BTX receptor was also determined in sartorius muscle (see table 3). Incubation of intact muscle with  $2 \times 10^{-8}$  M [ $^{125}$ I]  $\alpha$ -BTX (for 40 hrs at 4°C)

Figure 23. Time course of  $\alpha$ -BTX receptor extraction from sartorius muscle by 2% Triton X-100. Whole intact sartorius muscle which had been prelabelled with [ $^{125}$ I]  $\alpha$ -BTX as described in fig. 22 was extracted in 2% Triton X-100. Extraction was carried out by incubation with the detergent of either homogenized ( ● ) or intact ( ■ ) muscles at 4° C. After the desired period of time the extraction medium was removed by centrifugation in a Beckman microfuge for 1 min at 4° C. An aliquot of the supernatant was assayed for receptor bound toxin and the number of fmoles bound per muscle was determined. Values for toxin binding extracted from intact muscle are the average ( $\pm$ S.E.M.) of 4 individual sartorii.

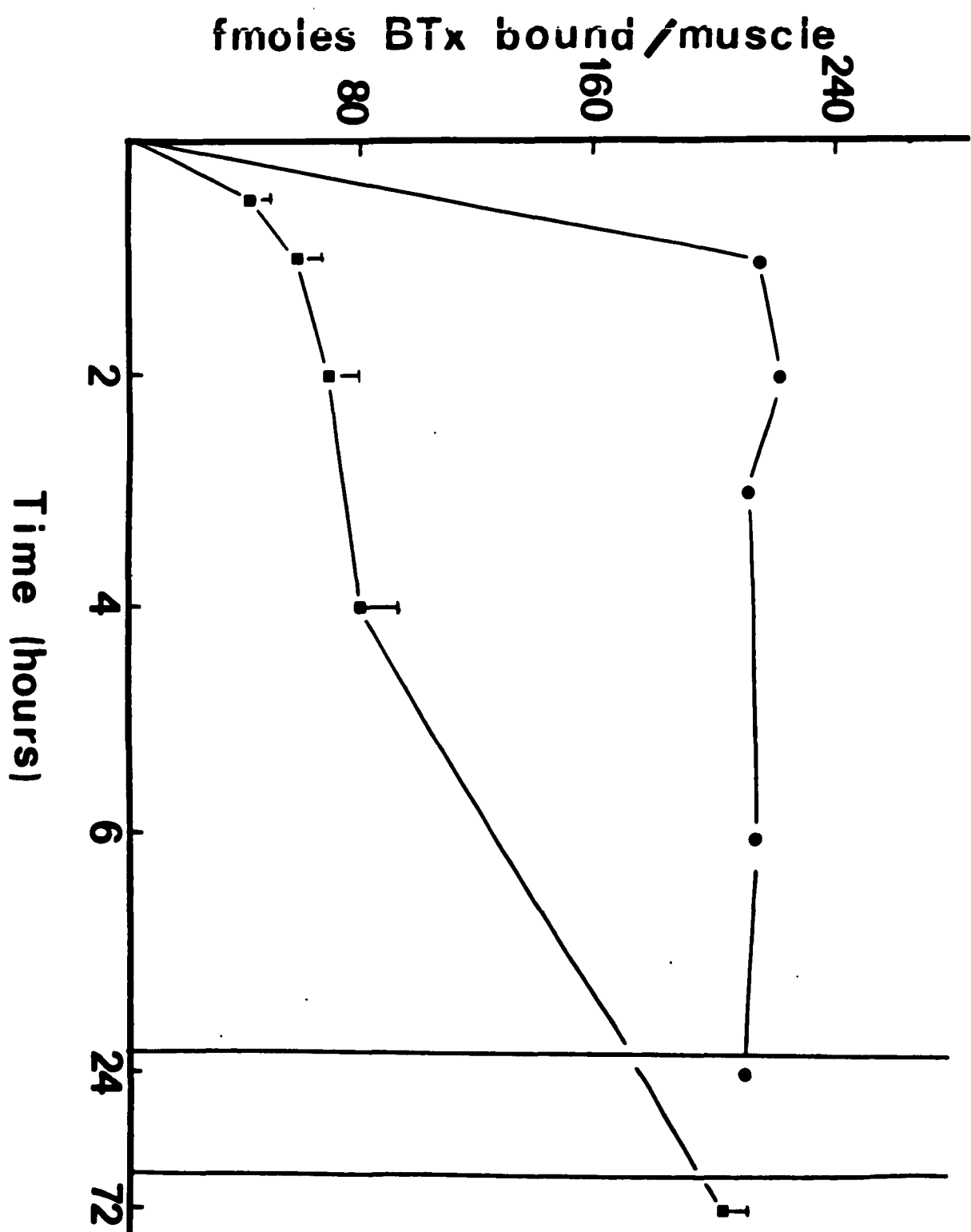


Table 3. Distribution of  $\alpha$ -BTX binding in whole frog sartorius muscle.

| Intact muscle <sup>a</sup>                       |                                                   |                                                |                                               |    |
|--------------------------------------------------|---------------------------------------------------|------------------------------------------------|-----------------------------------------------|----|
| incubation medium:                               | External BTX sites <sup>b</sup><br>(fmole/muscle) | Total BTX sites <sup>c</sup><br>(fmole/muscle) | Internal (hidden)<br>BTX sites (fmole/muscle) | n  |
| $2 \times 10^{-8}$ M [ $^{125}$ I] BTX           | $184.0 \pm 10.4$                                  | $221.9 \pm 9.6^*$                              | $29.8 \pm 9.6^d$                              | 22 |
| $2 \times 10^{-8}$ M unlabelled<br>$\alpha$ -BTX |                                                   |                                                | $21.6 \pm 12.0^e$                             | 20 |
| $4 \times 10^{-7}$ M unlabelled<br>$\alpha$ -BTX |                                                   |                                                | $22.3 \pm 6.1^e$                              | 6  |

\*Significantly different ( $P < .01$ ) from number of external toxin sites per muscle.

- All muscles were incubated in amphibian ringers soln (.65ml/muscle) containing the indicated conc. of  $\alpha$ -BTX for 40 hours at 4°C.
- External toxin binding was determined as described in the legend of figure 21.
- Total toxin binding was determined by Con A assay of the solubilized receptor after labelling with  $2 \times 10^{-8}$  M [ $^{125}$ I]  $\alpha$ -BTX overnight at 4°C.
- Calculated from the difference between total and externally bound [ $^{125}$ I]  $\alpha$ -BTX.
- Determined from Con A assay of solubilized receptor after labelling with  $2 \times 10^{-8}$  M [ $^{125}$ I]  $\alpha$ -BTX overnight at 4°C.

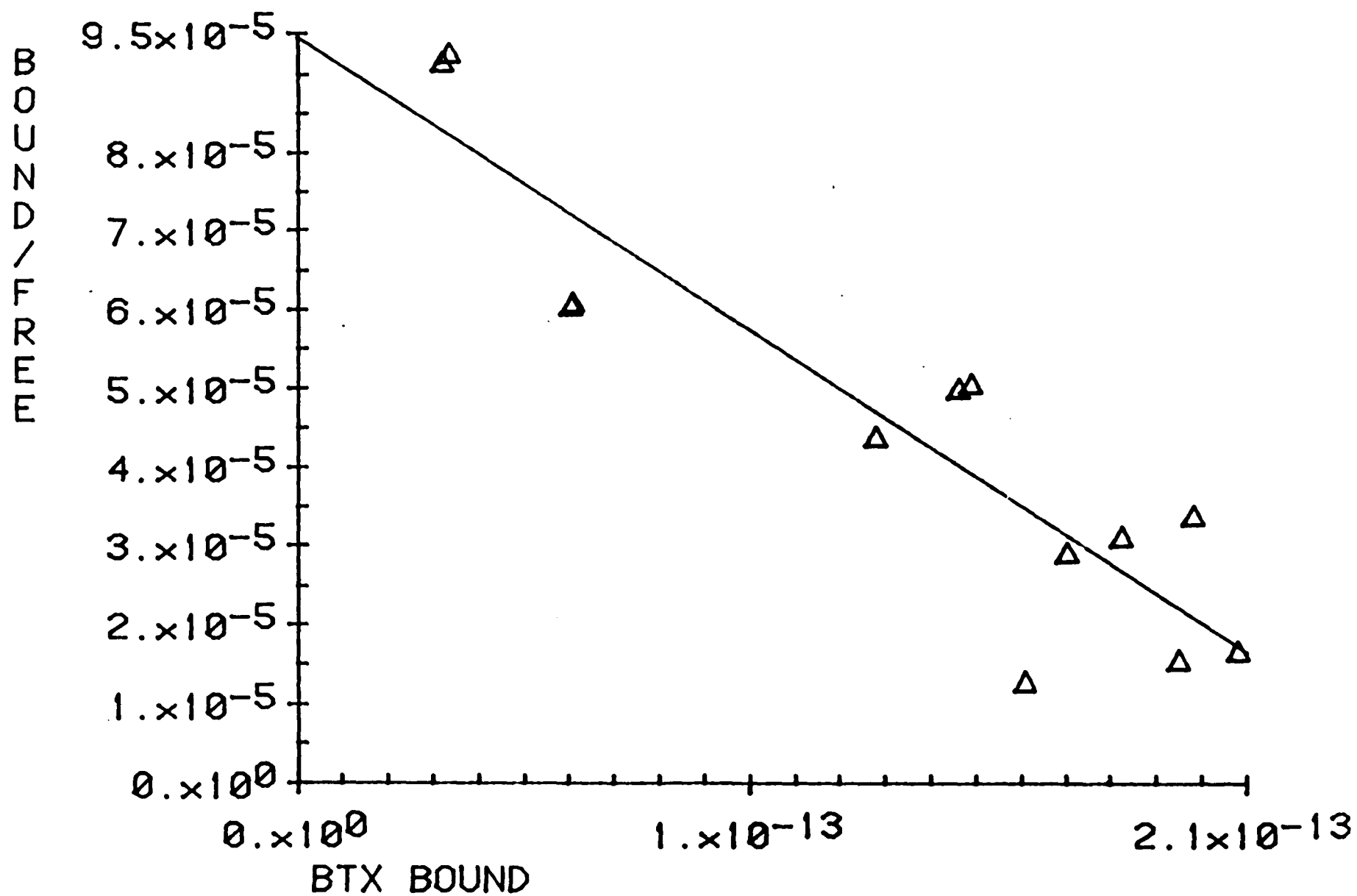
results in  $184.0 \pm 10.4$  fmoles of receptor bound toxin per muscle. Following homogenization and extraction, relabelling of the extract with  $2 \times 10^{-8}$  M [ $^{125}$ I]  $\alpha$ -BTX results in a significant increase ( $P < 0.01$ ) to a total of  $221.9 \pm 9.6$  fmoles of bound toxin per muscle, i.e.,  $29.8 \pm 9.6$  fmoles of sites are made available by solubilization. In agreement with this result an additional  $21.6 \pm 12.0$  fmoles of  $I^{125}$  toxin binding to internal (intracellular) receptor per muscle were measured in triton extracted receptor from muscles in which externally facing toxin binding sites had been masked by previous incubation of the intact muscle with unlabelled toxin ( $2 \times 10^{-8}$  M for 40 hrs at  $4^\circ\text{C}$ ). This small population of the total receptors ( $9.7 \pm 5.4\%$ ) is truly intracellular and not the result of a slow equilibration of toxin with a subfraction of extracellular sites. This is indicated by the observation that when intact muscle is incubated with 20x the concentration of cold toxin used above ( $4 \times 10^{-7}$  M) the same number of intracellular receptor sites are obtained ( $22.3 \pm 6.1$  fmoles/muscle) by relabelling of the triton extract with [ $I^{125}$ ] toxin. These results are consistent with observations demonstrating that approximately 15% of the total nAChR has an intracellular location as determined by autoradiography (Devreotes and Fambrough, 1975).

#### 14. Characterization of [ $^{125}$ I] $\alpha$ -BTX binding to intact sartorius muscle.

The affinity of [ $^{125}$ I]  $\alpha$ -BTX binding to intact sartorius muscle, the number of sites present per muscle, and the effect of DTT reduction and DPA alkylation on these parameters have been studied. The binding of [ $^{125}$ I]  $\alpha$ -BTX to untreated muscle, as assessed by the Con A column assay, can be described by a single hyperbolic function. Fig. 24 shows the binding as a function of the  $\alpha$ -BTX concentration under standard conditions (40 hr incubation at  $4^\circ\text{C}$ ; see Materials and Methods

Figure 24. Scatchard analysis of [ $^{125}$ I]  $\alpha$ -BTX binding to intact untreated sartorius muscle. Groups of 3 muscles were incubated at 4° C in amphibian Ringer's solution containing varying concentrations of [ $^{125}$ I]  $\alpha$ -BTX (see Materials and Methods section for standard conditions). Each muscle was assayed individually for bound toxin by the Con A column method. Analysis of results by the method of Scatchard can be fit to a single straight line ( $r=0.84$ ) and is consistent with a single class of binding sites having a  $B_{\max}$  of  $254.4 \pm 16.1$  fmoles/muscle and an apparent  $K_d$  of 2.68nM.

# UNTREATED



section). Scatchard analysis indicates a single class of binding sites with  $B_{\max}$  of  $254 \pm 16$  fmoles and an apparent  $K_d$  of  $2.68 \text{ nM}$ . Similar toxin binding characteristics are observed with intact DTT reduced sartorius muscles ( $2 \text{ mM}$  DTT for 20 min at  $23^\circ \text{C}$ ). A single class of binding sites with a  $B_{\max}$  of  $225 \pm 20$  fmoles/muscle and an apparent  $K_d$  of  $3.77 \text{ nM}$  was found (fig. 25). DPA alkylation of muscle, previously reduced with DTT, causes a significantly diminished affinity of [ $^{125}\text{I}$ ]  $\alpha$ -BTX for the receptor (see fig. 26) although the number of binding sites per muscle is unchanged by the treatment ( $257 \pm 23$  fmoles). A greater than 10-fold increase in the apparent  $K_d$  is obtained following DPA alkylation,  $30.1 \text{ nM}$  in contrast to  $2.68 \text{ nM}$  for control tissue. Prior reduction of the muscle is a necessary prerequisite for this effect of DPA on toxin binding. Similarly, it should be noted that the same is true for DPA to be able to produce an irreversible contraction. When the sequence required for DPA alkylation is reversed, i.e., DPA treatment before DTT reduction, no diminution of  $\alpha$ -BTX binding affinity is observed (fig. 27). Under these conditions both the apparent  $K_d$  ( $2.18 \text{ nM}$ ) and the total number of sites ( $235 \pm 15$  fmoles/muscle) for  $\alpha$ -BTX binding is found to be unchanged relative to untreated muscle. Experimental results obtained from replicate experiments of those described in fig. 24-27 are summarized in table 4.

N-methyl, N-benzyl aziridinium is a highly reactive structural analogue of DPA which can behave as an alkylating agent at nucleophilic sites (Rosen, Ehrenpreis and Karoutsou, 1973). However, unlike DPA, MBA does not interact with nicotinic receptors. At very high doses ( $10 \text{ mM}$ ), MBA does cause a contracture of the frog rectus preparation. However, this action of MBA is not inhibited by pre- or post-treatment with  $\alpha$ -BTX

**Figure 25.** Scatchard analysis of [ $^{125}$ I]  $\alpha$ -BTX binding to intact DTT reduced sartorius muscle. Freshly excised muscles were incubated in 2mM DTT in amphibian Ringer's solution for 20 min at 23° C prior to toxin labelling and Con A assay as described in fig. 24. Analysis of results by the method of Scatchard gave a straight line ( $r=.84$ ), consistent with a single class of binding sites with  $B_{\max}$  of  $225 \pm 20$  fmoles/muscle and an apparent  $K_d$  of 3.77nM.

# DTT TREATMENT

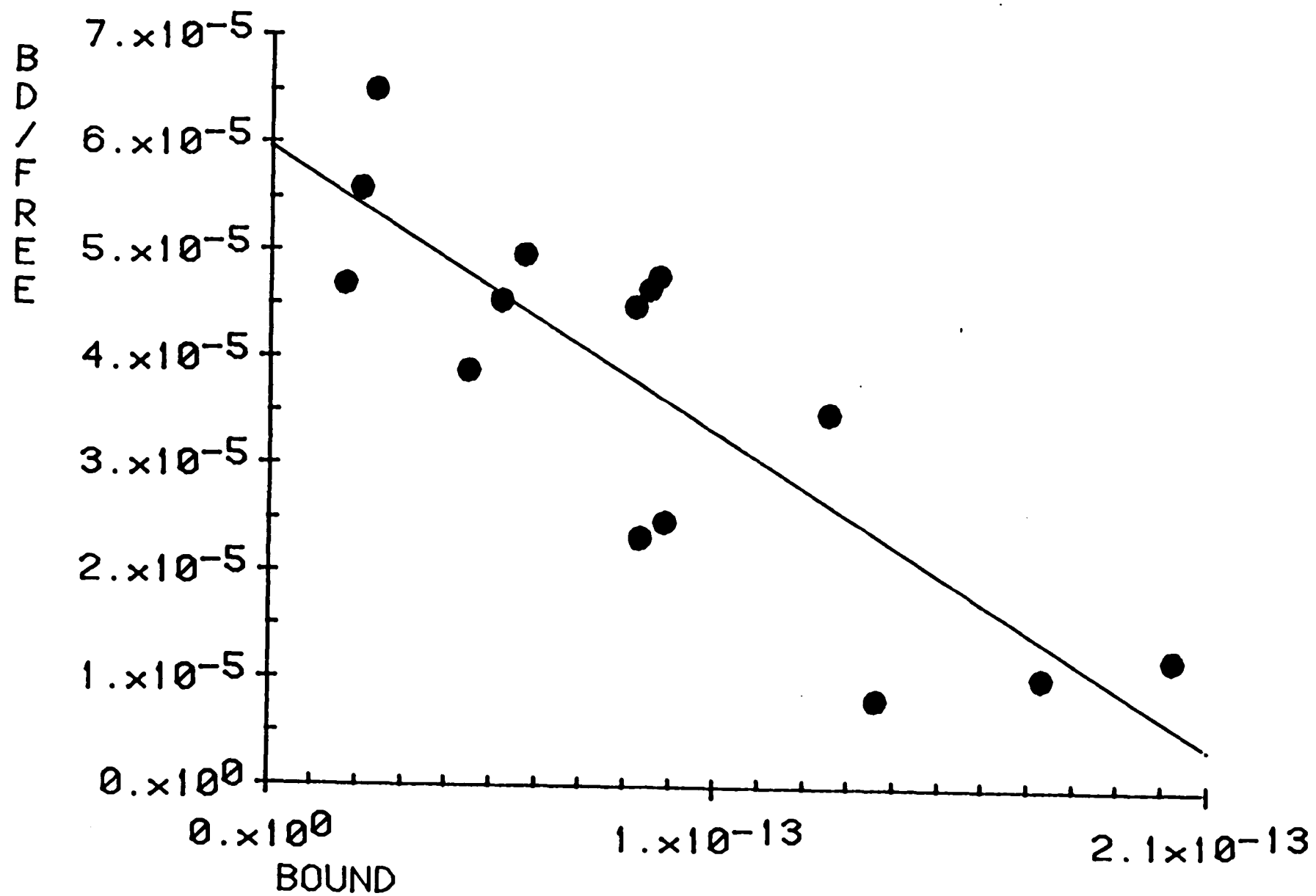


Figure 26. Scatchard analysis of [ $^{125}$ I]  $\alpha$ -BTX binding to intact DPA alkylated sartorius muscle. Freshly excised muscles were incubated with 2mM DTT in Ringer's solution for 20 min at 23° C and washed followed by DPA treatment and a second wash (see Materials and Methods for details). Muscles were then incubated with varying concentrations of [ $^{125}$ I]  $\alpha$ -BTX and the amount of receptor-toxin complex determined as described in the Materials and Methods section 2E. Analysis of results by the method of Scatchard gave a straight line ( $r=.87$ ) indicating a single class of binding sites equivalent in number with that obtained for untreated or DTT reduced muscle ( $B_{\max}=257\pm23$  fmoles/muscle) but having a reduced affinity for toxin (apparent  $K_d = 30.1$ nm).

# DTT FOLLOWED BY DPA TREATMENT

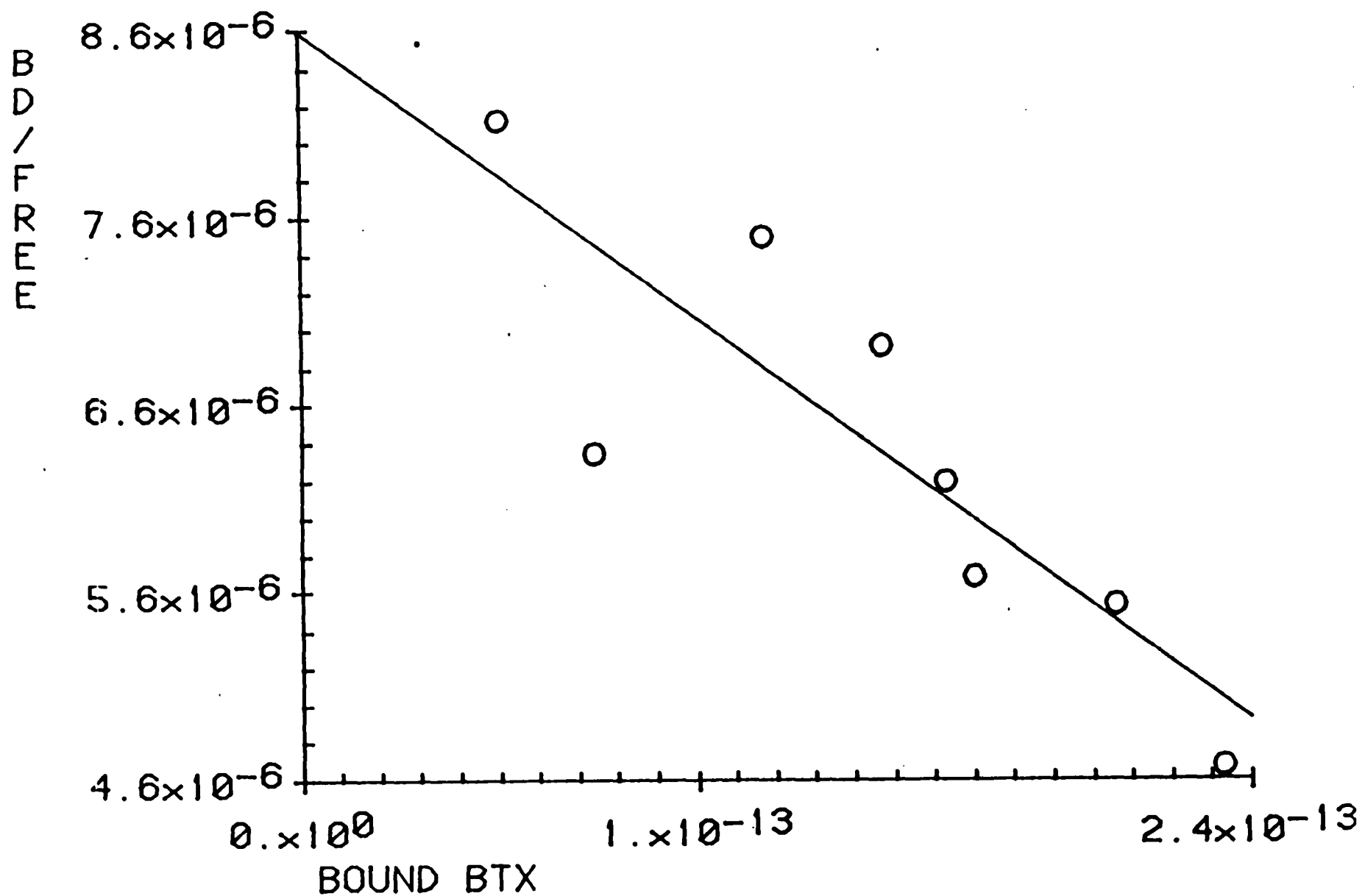


Figure 27. Scatchard analysis of [ $^{125}$ I]  $\alpha$ -BTX binding to intact sartorius muscle that had been alkylated with DPA prior to reduction with DTT. Experimental details are identical to those employed for the experiment described in fig. 26 with the exception that the order of muscle pretreatment was reversed. Scatchard analysis of the results indicates a single class of sites ( $r=.94$ ) which has a  $B_{\max}$  not significantly different from that of untreated or DTT treated muscle ( $235 \pm 15$  fmoles/muscle) and a similar apparent  $K_d$  (2.18nM).

# DPA TREATMENT FOLLOWED BY DTT TREATMENT

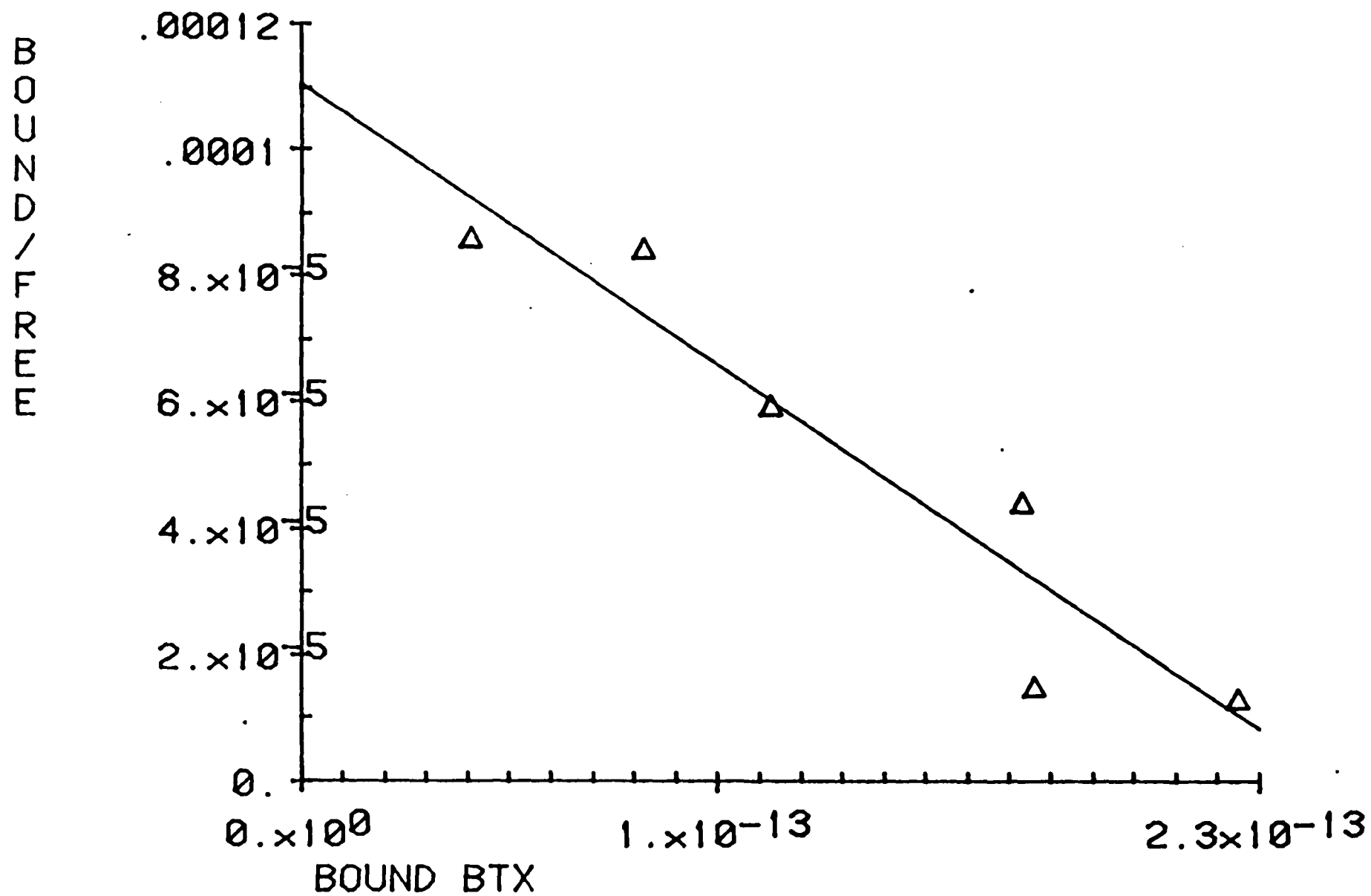


Table 4.  $[^{125}\text{I}]\alpha\text{-BTX}$  binding to intact sartorius muscle.

| TREATMENT                                                      | Apparent $K_d$ (nM) <sup>a</sup> | Bmax (fmol) <sup>a</sup> | n |
|----------------------------------------------------------------|----------------------------------|--------------------------|---|
| 1) untreated <sup>b</sup>                                      | $2.50 \pm .26$                   | $239.3 \pm 21.3$         | 3 |
| 2) DTT reduced <sup>b</sup>                                    | $3.45 \pm .71$                   | $232.0 \pm 12.0$         | 4 |
| 3) DTT reduced <sup>b</sup><br>followed by<br>DPA alkylation   | $45.8 \pm 13.8$                  | $240.0 \pm 16.0$         | 3 |
| 4) DPA alkylation <sup>b</sup><br>followed by DTT<br>reduction | $2.18 \pm .21$                   | $235 \pm 15$             | 1 |
| 5) DTT reduction <sup>c</sup><br>followed by MBA<br>alkylation | $2.14 \pm .23$                   | $210 \pm 6.0$            | 1 |

a. Mean of values derived from Scatchard analysis of n experiments.

b. Experimental protocol for 1-4 is described in figs. 23-26, respectively at 23°C.

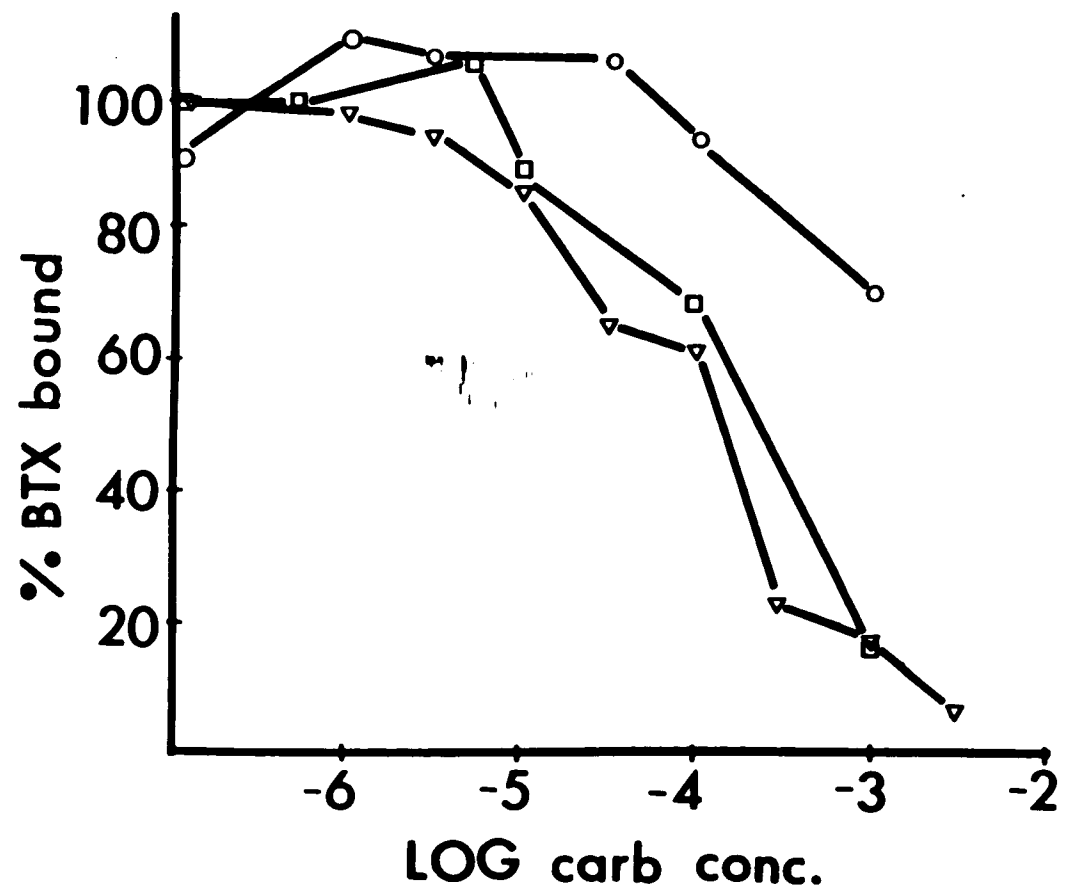
c. DTT reduction is as described in fig. 24, followed by 1mM MBA incubation for 20 minutes.

and is not dependent on DTT reduction of the muscle. This effect of MBA is believed to result from a non-specific action since other sulfhydryl reagents such as phenylmercuric acetate, N-ethyl maleimide, iodoacetamide, p-chloro-mercuri benzoate and o-iodoso-benzoate all have been shown to result in a similar contracture without involvement of nAChR (Chang et al., 1970). As indicated in Table 4, MBA treatment of DTT reduced muscle followed by washout is ineffective as an irreversible agonist and also results in no change in either the affinity or number of sites available for [ $^{125}$ I]  $\alpha$ -BTX binding; the apparent  $K_d$  was  $2.14 \pm 0.23$  nM, and the  $B_{max}$  was  $210 \pm 6.0$  fmoles. These findings are consistent with the suggestion that the effect of DPA alkylation of reduced muscle on toxin binding affinity results from a specific reaction of DPA with the receptor channel complex.

15. Competition by carbachol, dTC and PCP for [ $^{125}$ I]  $\alpha$ -BTX binding to intact sartorius muscle.

The ability of various ligands to compete for [ $^{125}$ I]  $\alpha$ -BTX binding to whole intact sartorius muscle was studied in untreated, DTT reduced and DPA alkylated muscle. The toxin concentration used for competition was 3 nM for both untreated and DTT reduced muscle and 30 nM for DPA alkylated muscle, i.e., approximately equal to the apparent  $K_d$  in each case. The results obtained for carbachol, shown in fig. 28, indicate that in DPA alkylated muscle carbachol has a decreased ability to inhibit toxin binding relative to the control muscles. Similar  $EC_{50}$ 's for carbachol are obtained in the untreated and DTT reduced muscle ( $108 \pm 28.2$   $\mu$ M and  $80.5 \pm 21.3$   $\mu$ M, respectively). Since only 30% inhibition of toxin binding is the maximum effect attained by 1 mM carbachol (the highest concentration employed) in DPA treated sartorius, only an

Figure 28. Competition of carbachol for  $\alpha$ -BTX binding to whole sartorius muscle. Binding experiments in intact sartorius muscle were performed as described in the Materials and Methods section. Groups of 3 muscles were preincubated for 8 hrs at 4° C in 1.5 ml amphibian Ringer's containing the desired concentration of carbachol. Toxin binding reaction was begun by addition of [ $^{125}$ I]  $\alpha$ -BTX to give a final concentration of 3nM (30nM for DPA alkylated muscle) and allowed to proceed for 40 hrs at 4° C. Individual muscles were washed, extracted in Triton X-100 and duplicate aliquots assayed by the Con A method. Formation of receptor-toxin complex is plotted as a function of [carbachol] for untreated (squares), DTT reduced (triangles), and DPA alkylated (circles) muscle. Each point represents the mean of 3 individual muscle determinations minus the nonspecific binding (determined by simultaneous incubation of a group of muscles with a 33-fold excess of unlabelled toxin ( $1 \times 10^{-7}$  M). The  $EC_{50}$  for carbachol inhibition of BTX binding is calculated to be  $108.0 \pm 28.2$  and  $80.5 \pm 21.3 \mu$ M for untreated and DTT reduced muscle, respectively. DPA alkylation results in an  $EC_{50}$  greater than 1mM.



approximate  $EC_{50}$  can be calculated, because of the higher degree of error at smaller degrees of inhibition.

In contrast to the results with carbachol, dTC was found to be an equipotent inhibitor of [ $^{125}$ I]  $\alpha$ -BTX binding in untreated, DTT reduced and DPA alkylated intact sartorius muscle (fig. 29).  $EC_{50}$  values determined for curare inhibition of  $\alpha$ -BTX binding in these tissues are 23.5  $\mu$ M, 25.4  $\mu$ M, and 18.6  $\mu$ M, respectively. These findings indicate that DPA alkylation of the receptor selectively interferes with the ability of carbachol, an agonist, to compete for toxin binding, while it is without effect on the antagonist dTC to compete for binding. Results of these competition binding experiments in intact muscle are summarized in table 5. PCP was found to inhibit toxin binding only at very high concentrations (mM) and a study of this action was limited by its maximum solubility in water at physiological pH ( $2 \times 10^{-2}$  M). It is clear, however, that the  $EC_{50}$  of PCP is in excess of 1 mM in all three of the muscle preparations used — much greater than its  $EC_{50}$  as an antagonist of carbachol responses.

#### 16. Characterization of [ $^{125}$ I] $\alpha$ -BTX binding to sartorius muscle extracts.

The characteristics of the binding of  $\alpha$ -BTX to receptors solubilized by Triton X-100 from muscle preparations was studied for comparison with results obtained for binding to intact muscle (membrane-bound receptors). Experiments were performed using extracts containing the solubilized nAChR prepared from intact sartorius muscle which were untreated or previously reduced by DTT and DPA alkylated. Muscle extracts were incubated with a range of [ $^{125}$ I]  $\alpha$ -BTX concentrations at 4°C and the specific binding measured after a fixed time interval (24

**Figure 29.** Competition of dTC for  $\alpha$ -BTX binding to whole sartorius muscle. Binding experiments in intact sartorius muscle were performed as described in the Materials and Methods section. Groups of 3 muscles were preincubated for 8 hrs at 4° C in 1.5 ml amphibian Ringer's containing the desired concentration of d-tubo. The toxin binding reaction was begun by addition of [ $^{125}$ I]  $\alpha$ -BTX to give a final concentration of 3nM in the case of untreated and DTT reduced muscle and 30nM for DPA alkylated muscle. The reaction was allowed to proceed for 40 hrs at 4° C after which time the muscles were washed, extracted in Triton X-100 and duplicate aliquots subjected to the Con A assay. Receptor-toxin complex is plotted as a function of dTC concentration for untreated (squares), DTT reduced (circles), and DPA alkylated (triangles). Each point represents the mean of 3 individual muscle determinations minus the nonspecific binding (determined by simultaneous incubation of a group of muscles with a 33-fold excess of unlabelled  $\alpha$ -BTX). The EC<sub>50</sub> for dTC inhibition of  $\alpha$ -BTX binding was 22.3 $\pm$ 3.2 $\mu$ M, 25.4 $\pm$ 7.3 $\mu$ M, and 18.6 $\pm$ 3.9 $\mu$ M for untreated, DTT reduced, and DPA alkylated muscle, respectively.

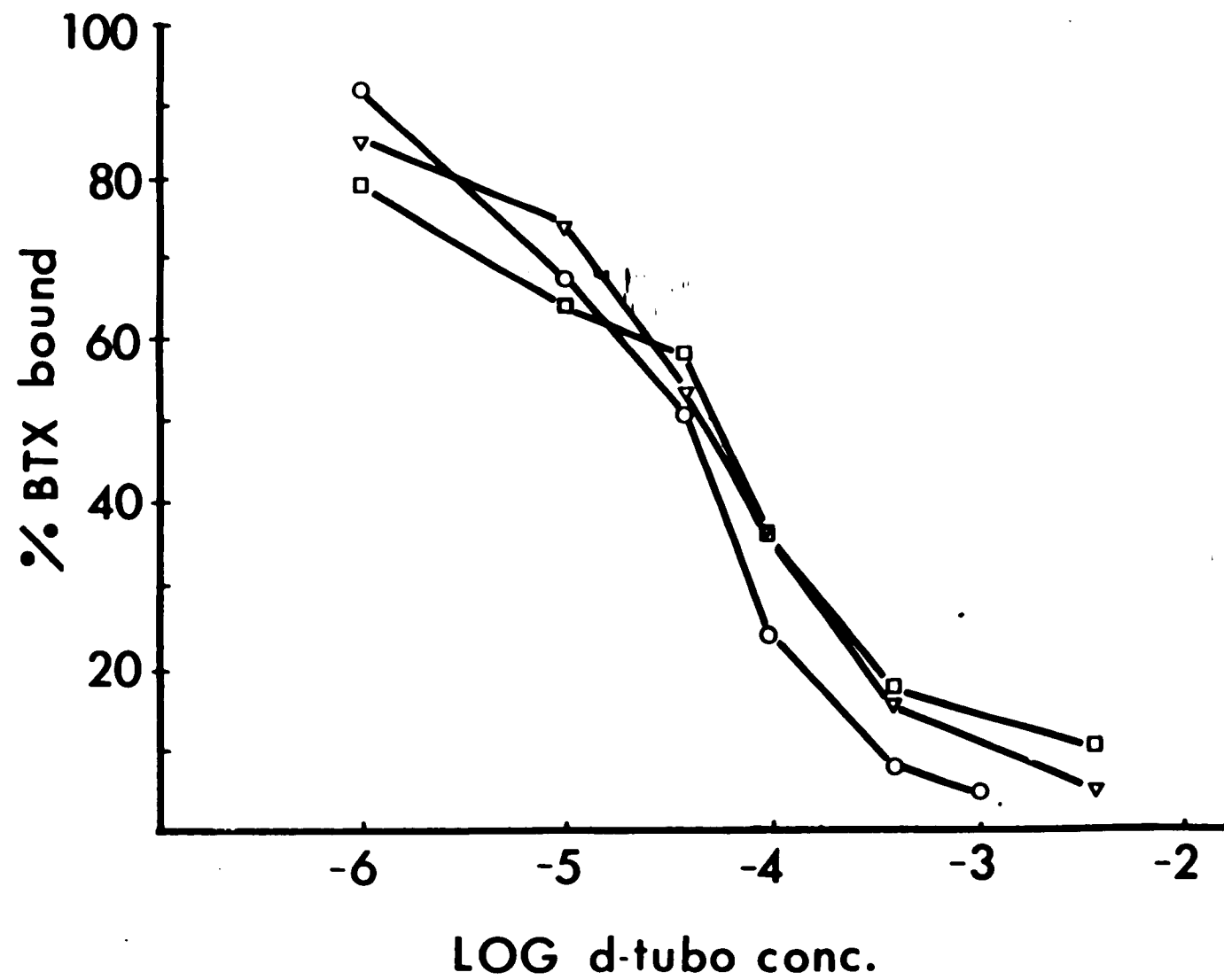


Table 5. Ability of ligands to compete for [ $^{125}$ I]  $\alpha$ -BTX binding to intact sartorius muscle (membrane bound AChR showing one set of BTX binding sites by apparent equilibrium binding).

IC<sub>50</sub> for toxin binding inhibition<sup>1</sup>

|                            | Carbachol (uM)   | dTC (uM)       | PCP (uM) |
|----------------------------|------------------|----------------|----------|
| Untreated                  | 135.3 $\pm$ 31.6 | 23.0 $\pm$ 9.3 | >1000    |
|                            | (n=3)            | 22.5 $\pm$ 3.2 | (n=3)    |
| DTT reduced <sup>2</sup>   | 142.7 $\pm$ 28.2 | 25.4 $\pm$ 7.3 | >1000    |
|                            | 80.5 $\pm$ 21.3  | 33.0 $\pm$ 5.4 | (n=2)    |
| DPA alkylated <sup>3</sup> | >1000            | 33.0 $\pm$ 7.1 | >1000    |
|                            | (n=2)            | 18.6 $\pm$ 3.9 | (n=2)    |

1. Protocol is as described in Methods, 2F (3nM [ $^{125}$ I]  $\alpha$ -BTX for 48 hours at 4°C.)

2. DTT reduction is as described in figure 25.

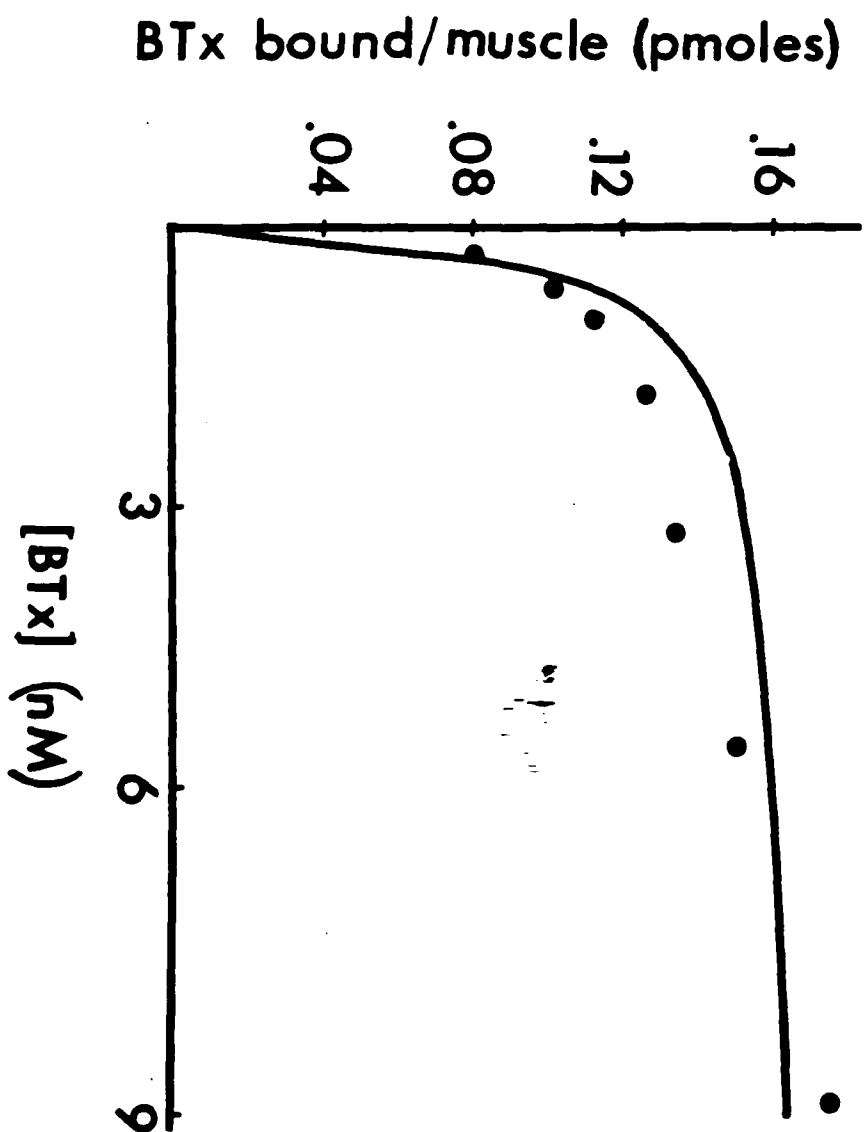
3. DPA alkylation is as described in figure 26.

hrs). Whereas the binding of toxin to intact muscle was attributable in each kind of preparation to a single class of binding sites (linear Scatchard plots), the toxin binding characteristics of solubilized muscle appear more complex. When data obtained for specific binding to untreated muscle extract are plotted as a function of toxin concentration, a systematic deviation is observed from the expected hyperbola through the data points (fig. 30A). Likewise, graphic analyses of the data by both double reciprocal plot and by the method of Scatchard (fig. 30B and C), in which all points are expected to fall on a straight line, for a single class of independent binding sites, show deviations from linearity. Similar results have been obtained in three individual experiments, all of which suggest the presence of a higher and lower affinity binding site/state for  $\alpha$ -BTX binding. In common with untreated muscle, the toxin binding behavior of solubilized nAChR from DTT reduced and DPA alkylated muscle cannot be adequately described by a single class of independent sites (figs. 31 and 32, respectively).

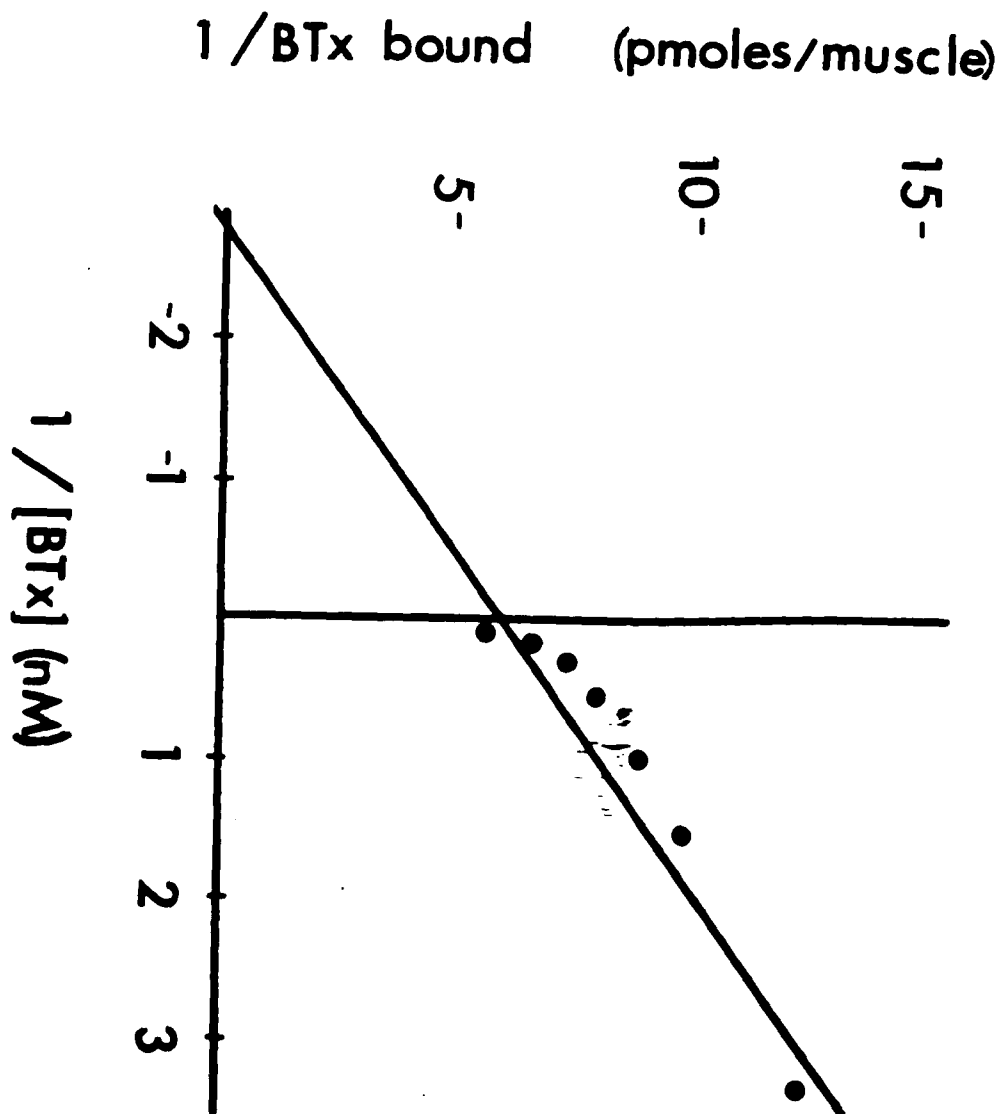
Scatchard analysis of  $\alpha$ -BTX bound to muscle extract over the limited concentration range of  $3 \times 10^{-10}$  to  $5 \times 10^{-9}$  M yields a straight line which represents what appears to be the high affinity toxin binding component. Thus at low concentrations of toxin or at short incubation times this component of homogeneous toxin binding sites can be independently studied. At longer times the second set of sites can be studied in kinetic experiments. For comparison,  $\alpha$ -BTX binding to untreated, DTT reduced and DPA alkylated muscle extracts was analyzed in this restricted concentration range in order to detect any effect of the drug pretreatment on the affinity of toxin binding to one set of binding sites. No significant difference in the apparent  $K_d$  was observed

Figure 30.  $\alpha$ -BTX binding (24 hr) to solubilized nAChR from untreated muscle. The binding of [ $^{125}$ I]  $\alpha$ -BTX to solubilized untreated sartorius muscle nAChR was performed as described in the Materials and Methods section under standard (pseudoequilibrium) conditions. The muscle extract was incubated with the desired concentrations of labelled toxin for 24 hrs at 4° C and assayed in duplicate by the Con A assay for receptor-toxin complex. The range of toxin concentration was  $2 \times 10^{-10}$  M- $10^{-8}$  M. The data were analyzed graphically by the hyperbolic (A), double reciprocal (B) and Scatchard methods (C). The best fit hyperbola (A) and best fit straight lines (B and C) which were calculated by Prophet using non-linear regression analysis are drawn through the data points. Results suggest the presence of at least two binding sites of differing affinity for  $\alpha$ -BTX in the preparation.

A.



B.



C.

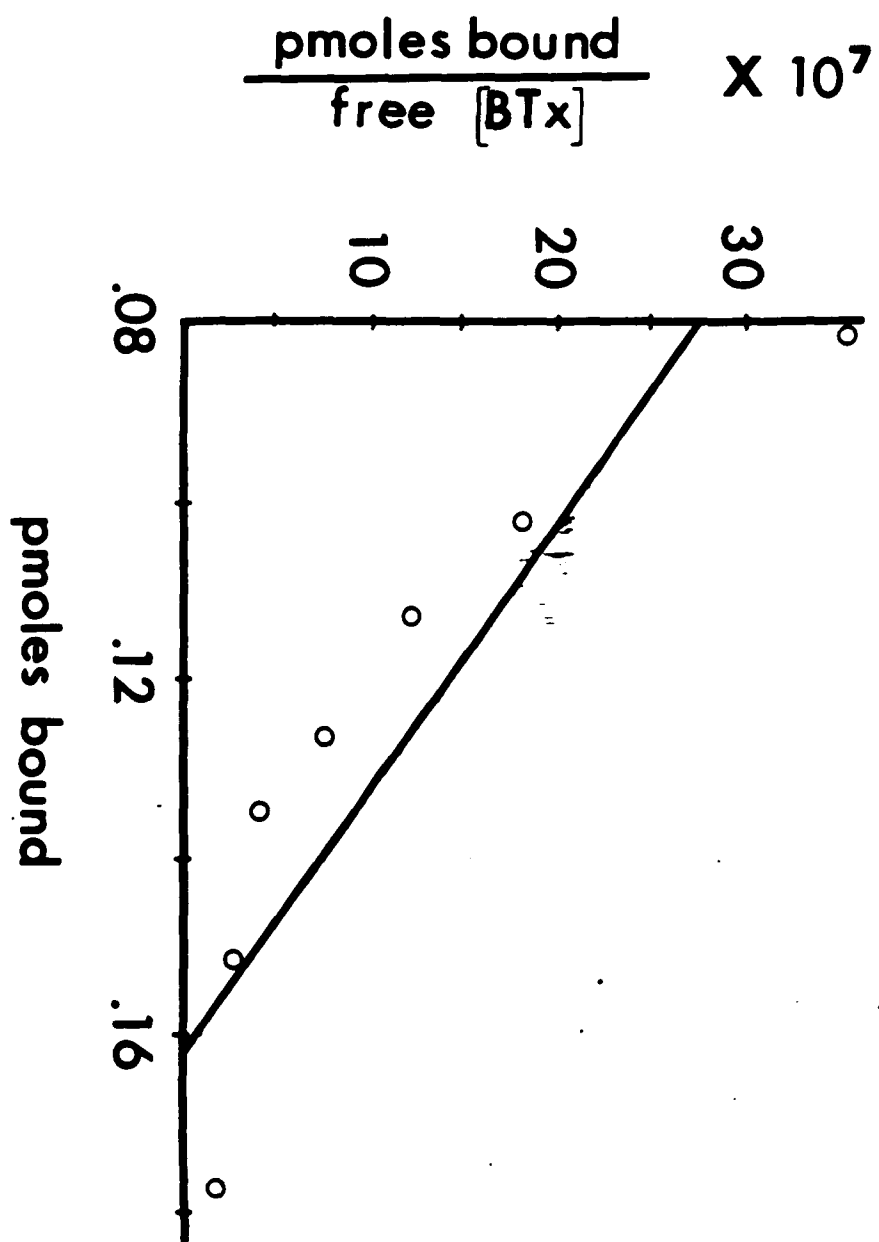


Figure 31. Scatchard analysis of [ $^{125}$ I]  $\alpha$ -BTX binding (24 hr) to solubilized nAChR after DTT reduction of intact muscle. Binding of [ $^{125}$ I]  $\alpha$ -BTX to nAChR extracted from DTT treated sartorius muscle was performed as described in the Materials and Methods section under standard (pseudoequilibrium) conditions. The muscle extract was incubated with the desired concentrations of labelled toxin for 24 hrs at 4° C and assayed in duplicate by the Con A assay for toxin-receptor complex. The data obtained in the range of toxin concentration from  $1 \times 10^{-10}$  -  $7 \times 10^{-8}$  M is analyzed by the method of Scatchard. The non-linear nature of the points obtained suggested the presence of multiple sites which exhibit different affinities for  $\alpha$ -BTX. The solid line represents best fit to the points assuming one set of sites.

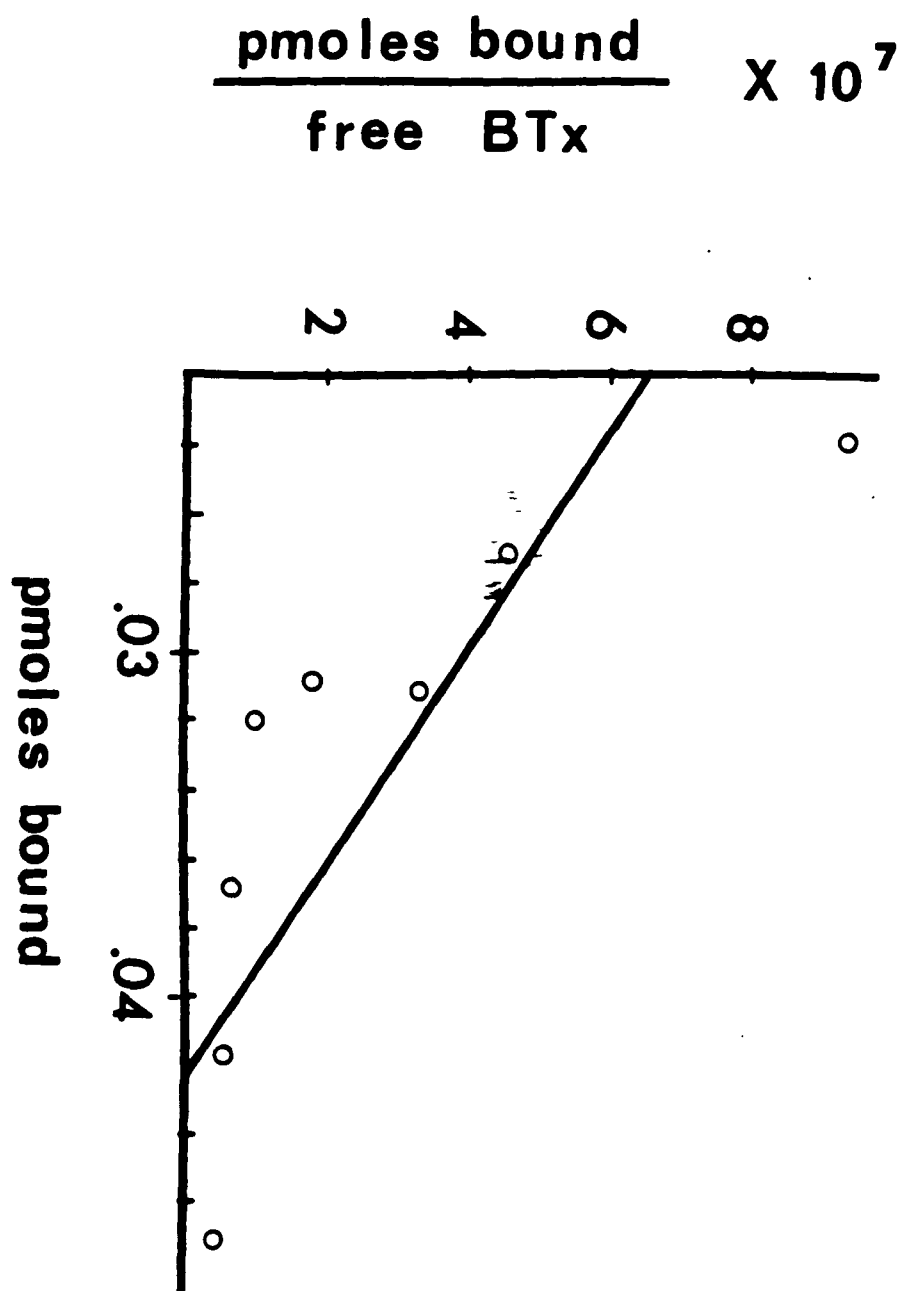
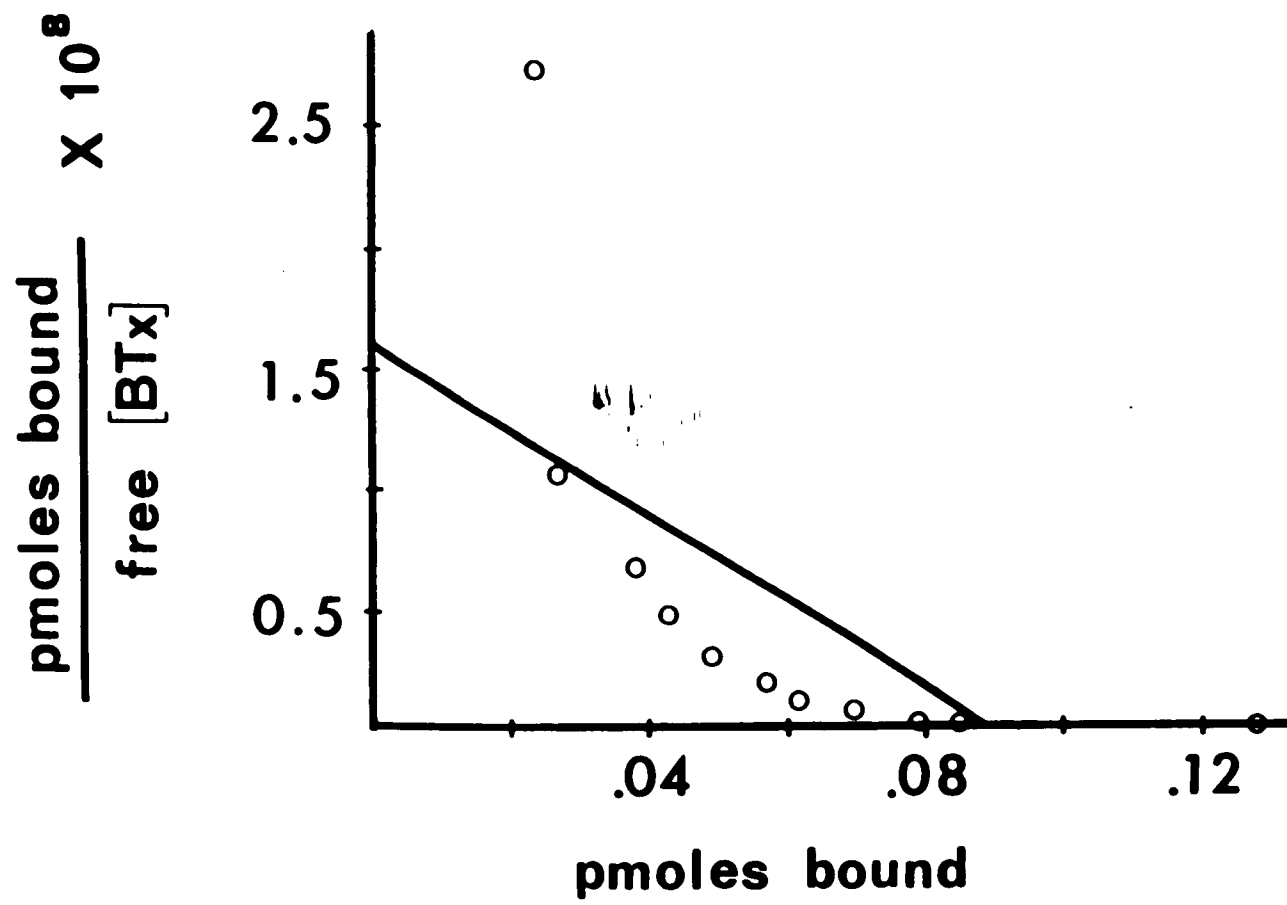


Figure 32. Scatchard analysis of  $\alpha$ -BTX binding to solubilized nAChR from DTT reduced and DPA alkylated muscle. Binding of [ $^{125}$ I]  $\alpha$ -BTX to solubilized muscle derived from DPA alkylated sartorius muscle was performed as described in the Materials and Methods section under standard (pseudoequilibrium) conditions. The muscle extract was incubated with the desired concentrations of labelled toxin for 24 hrs at 4° C and assayed in duplicate for toxin-receptor complex by the Con A assay. The data obtained in the range of toxin concentration from  $2 \times 10^{-10}$  -  $10^{-8}$  M were analyzed by the method of Scatchard. The non-linear nature of the data points obtained suggests, as for untreated and DTT treated muscle, the presence of two binding sites of differing affinity for  $\alpha$ -BTX.



resultant from these treatments; all values were in the range of  $2.3 \times 10^{-10}$  M (see table 6). This finding is in contrast to the observation that DPA treatment of the membrane-bound receptors results in a greater than 10-fold reduction of the affinity for  $\alpha$ -BTX in intact muscle. For soluble nAChR the concentration of high affinity  $\alpha$ -BTX binding sites is found not to be significantly altered by DTT-DPA treatment although the standard errors for these values are somewhat greater than for other toxin binding experiments.

17. Dissociation of bound [ $^{125}$ I]  $\alpha$ -BTX from muscle extract.

The time course of [ $^{125}$ I]  $\alpha$ -BTX dissociation from its receptor in detergent solubilized muscle is so slow that for all practical purposes binding is irreversible (fig. 33). After 21.15 and 92.4 hrs only 3% and 13.3% of the total receptor toxin complex, respectively, appears to have dissociated. These values represent dissociation plus any receptor degradation which would also be considered as dissociation and could contribute significantly after prolonged times. Computer analysis (Prophet Expfit program), assuming a single first order dissociation process, provides an upper limit for the dissociation rate constant of  $2.65 \pm 0.31 \times 10^{-5} \text{ min.}^{-1}$ .

18. Competition of carbachol, dTC and PCP for binding of [ $^{125}$ I]  $\alpha$ -BTX to nAChR extracted from untreated sartorius muscle extract.

Competition binding experiments were performed by incubation of the extract from untreated muscles for a fixed time interval with [ $^{125}$ I]  $\alpha$ -BTX in the presence of various concentrations of a competing ligand. As shown in figure 34 both dTC and carbachol are effective competitors for toxin binding in the concentration range over which they are physiologically active. IC<sub>50</sub>'s obtained in this experiment are 6.43  $\mu$ M

Table 6.  $K_d$  apparent for  $[^{125}\text{I}] \alpha\text{-BTX}$  binding to high affinity site in muscle extract<sup>1</sup> (solubilized AChR).

|                                             | Untreated<br>(n=3) | DTT treated      | DPA alkylated<br>(n=3) |
|---------------------------------------------|--------------------|------------------|------------------------|
| $K_{d_{app}}$ ( $\times 10^{-10}\text{M}$ ) | $2.24 \pm .014$    | $3.01$<br>$2.43$ | $2.78 \pm 1.25$        |
| # sites/<br>sartorius muscle<br>(fmoles)    | $86.4 \pm 28.6$    | $59.4$<br>$75.3$ | $82.3 \pm 23.7$        |
| Correlation<br>coefficient <sup>2</sup>     | $.969 \pm .014$    | $.963$<br>$.981$ | $.949 \pm .020$        |

1. Defined by a toxin concentration range  $3 \times 10^{-10}\text{M}$  to  $5 \times 10^{-9}\text{M}$  (6-8 toxin concentrations used for evaluation).
2. Correlation coefficient for best fit line to Scatchard analysis for single set of binding sites.

Figure 33. Kinetics of dissociation of  $\alpha$ -BTX from solubilized muscle nAChR. Triton X-100 solubilized muscle nAChR, derived from untreated sartorius muscle was labelled overnight with  $1 \times 10^{-8}$  M [ $^{125}$ I]  $\alpha$ -BTX. A 50-fold excess of unlabelled toxin (concentration =  $5 \times 10^{-7}$  M) was added at time 0, and samples from the reaction mixture at 4° C were assayed at various times over a 100 hr interval by the Con A method. Less than 15% dissociation of toxin (plus denaturation and proteolysis) occurred during this time interval. Computer analysis of these data (see data analysis section in Materials and Methods) gives a value of  $2.66 \times 10^{-5}$  min $^{-1}$ . The coefficient of linear regression,  $r^2$ , is 0.999.

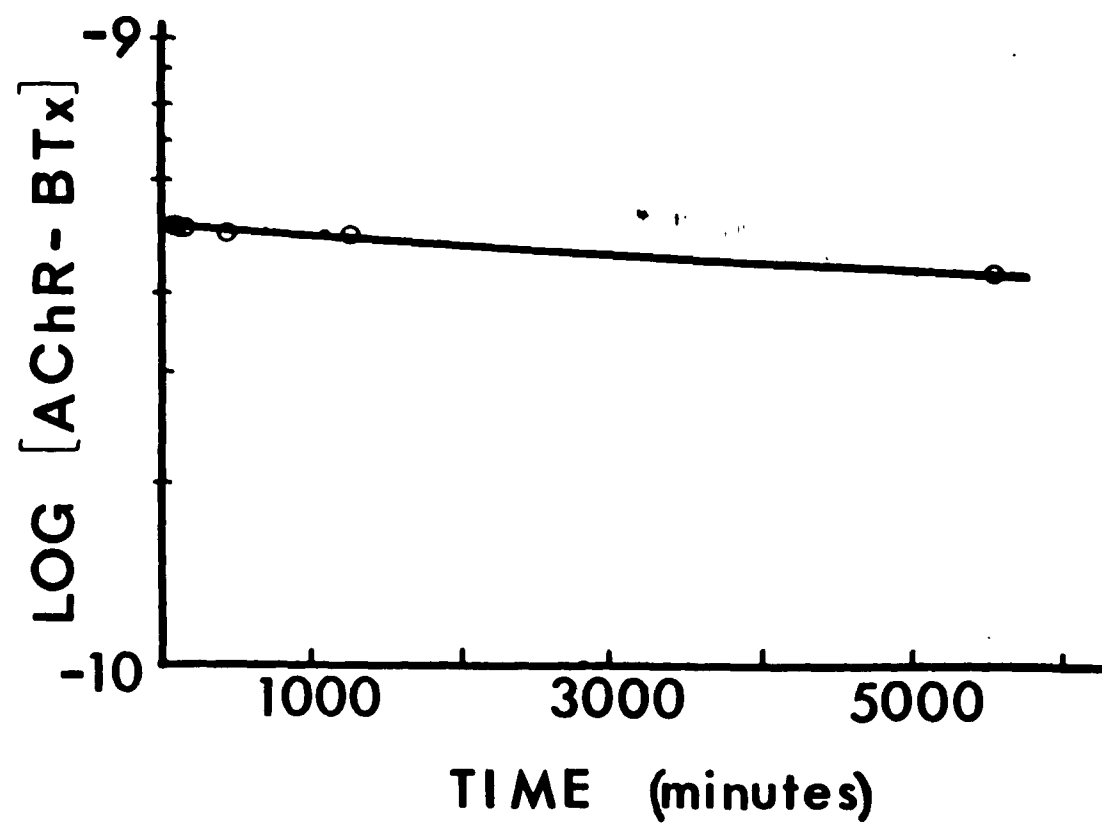
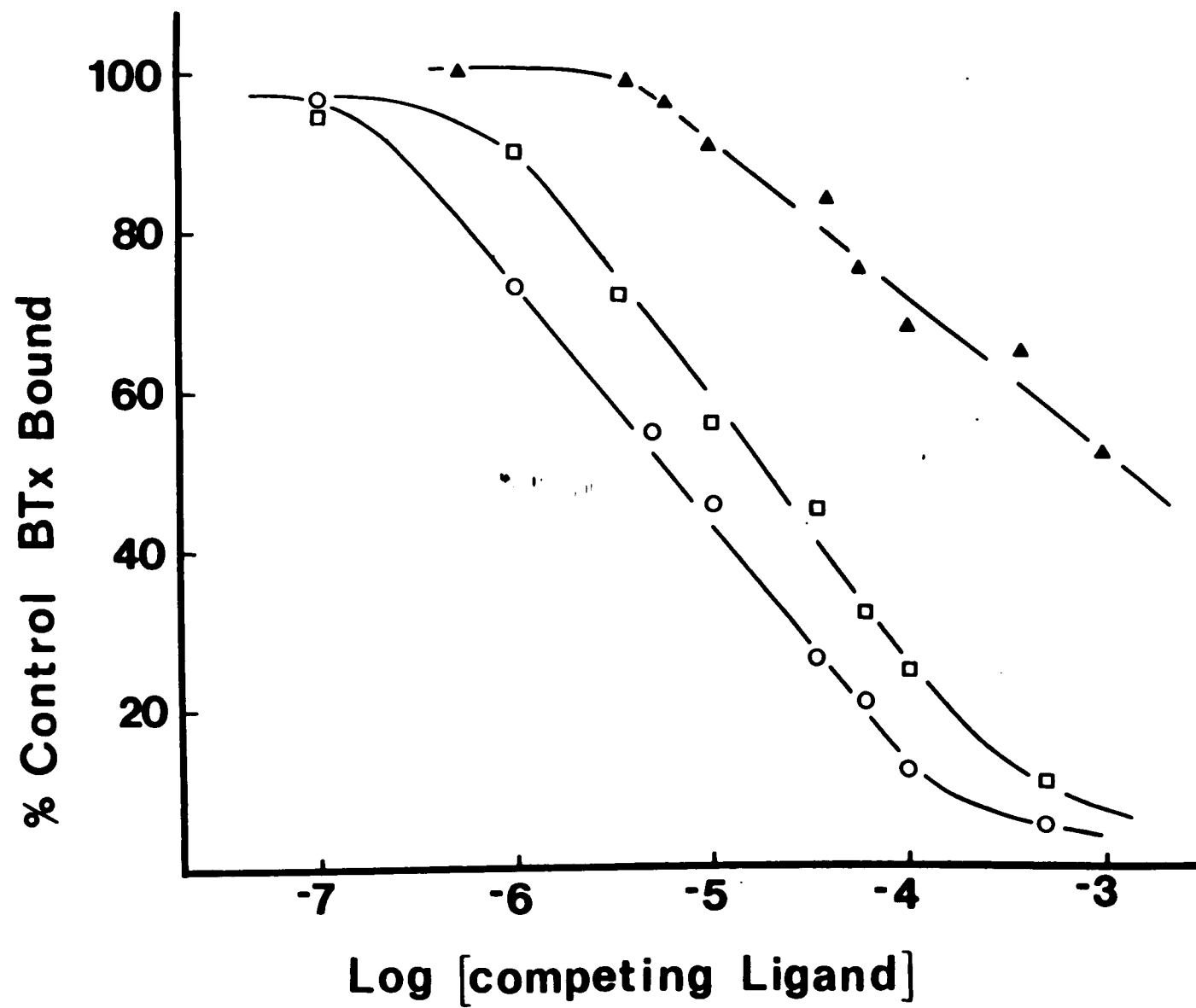


Figure 34. Competition of carbachol, dTC, and PCP for  $\alpha$ -BTX binding to untreated muscle extract (24 hrs). Aliquots of Triton X-100 solubilized sartorius muscle nAChR were preincubated for 60 min at 4° C with the desired concentration of competing ligand. The binding reaction was then started by addition to each sample of [ $^{125}$ I]  $\alpha$ -BTX to give a final concentration of 5nM. After 24 hrs at 4°C the [toxin-receptor complex] was determined by Con A assay of duplicate aliquots from each sample. The EC<sub>50</sub> for toxin inhibition was determined by the Prophet Logistic program to be 6.43 $\mu$ M (Hill coefficient=0.66) and 13.8 $\mu$ M (Hill coefficient=0.68) for dTC (circles) and carbachol (squares), respectively. Although 50% toxin inhibition was not quite achieved by the highest concentration of PCP (triangles) employed, extrapolation results in an EC<sub>50</sub> of 1.03mM.



with a Hill coefficient of 0.66) and  $13.8\mu\text{M}$  with a Hill coefficient of 0.68 for dTC and carbachol, respectively. In contrast, PCP was observed to compete for toxin binding only at a concentration range well in excess of that necessary for biological activity. The  $\text{IC}_{50}$  for PCP inhibition of the contractile response to carbachol is  $5\mu\text{M}$ ; the  $\text{IC}_{50}$  for inhibition of  $\alpha$ -BTX can only be estimated by extrapolation and is greater than  $1\text{mM}$ . Furthermore, the shallowness of the PCP curve for inhibition of toxin binding suggests an indirect, possibly allosteric, mode of action. Results obtained from replicate experiments for  $\text{IC}_{50}$  and Hill coefficient values of carbachol, dTC and PCP inhibition of toxin binding are summarized in table 7.

Although PCP was not found to be a potent competitor for toxin binding to the receptor, it does influence the toxin binding sites. The apparent affinity for carbachol and dTC to compete for  $\alpha$ -BTX binding was decreased by  $2 \times 10^{-5}\text{M}$  PCP. At this concentration, PCP itself causes a  $27.3 \pm 4.7\%$  inhibition in toxin binding under the conditions defined for the competition binding experiments. The results of a study of carbachol and dTC competition for the residual, approximately 73%, binding, indicate a significant decrease in the  $\text{IC}_{50}$  values for both these ligands (see figures 35 and 36). Also noted is an increase in the slope of these competition binding curves. Results which are summarized in table 7 indicate a 3.29-fold decrease in the ability of carbachol to compete for toxin in the presence of  $2 \times 10^{-5}\text{M}$  PCP (from  $18.9 \pm 6.3$  to  $62.2 \pm 14.0\mu\text{M}$ ,  $P < .01$ ). Additionally, in the presence of PCP a significant increase in the Hill coefficient for carbachol competition is observed to a value approaching 1.0 (from  $0.62 \pm 0.04$  to  $0.90 \pm 0.05$ ,  $P < .01$ ). These results suggest an allosteric action of PCP on the receptor affecting

Table 7. Inhibition of  $\alpha$ -BTX binding (5nM) by carbachol and by dTC in absence or presence of PCP in sartorius muscle extract.

|           |  | Treatment                     |                               |
|-----------|--|-------------------------------|-------------------------------|
|           |  | <u>Control</u>                | <u>200uM PCP</u>              |
|           |  | EC <sub>50</sub> (uM)*      n | EC <sub>50</sub> (uM)*      n |
| Carbachol |  | 18.9 $\pm$ 6.3      3         | 62.2 $\pm$ 14.0      4        |
| dTC       |  | 5.32 $\pm$ 1.14      3        | 11.6 $\pm$ 3.6      3         |
| PCP       |  | 1039      2<br>1017           |                               |

\*Values obtained using 5nM [<sup>125</sup>I]  $\alpha$ -BTX incubated 24 hours at 4°C. in the presence of competing ligand.

Figure 35. Competition of carbachol, in the presence or absence of PCP, for  $\alpha$ -BTX binding to solubilized nAChR. Varying concentrations of carbachol alone (open circles) or carbachol in the presence of  $2 \times 10^{-6}$  M PCP (filled circles) were preincubated for 60 min at 4°C with Triton extracted nAChR from sartorius muscle. Toxin labelling was done under conditions for the competition binding experiment as described in fig. 34. The reference point for 100% toxin binding was determined by the binding of toxin in the presence of PCP alone ( $2 \times 10^{-6}$  M) which is about 70% of that found in the absence of PCP. The  $IC_{50}$  values for carbachol inhibition of [ $^{125}$ I]  $\alpha$ -BTX binding were found to be 13.8  $\mu$ M (Hill coefficient=0.68) in the absence and 73.0  $\mu$ M (Hill coefficient=0.91) in the presence of PCP, respectively.

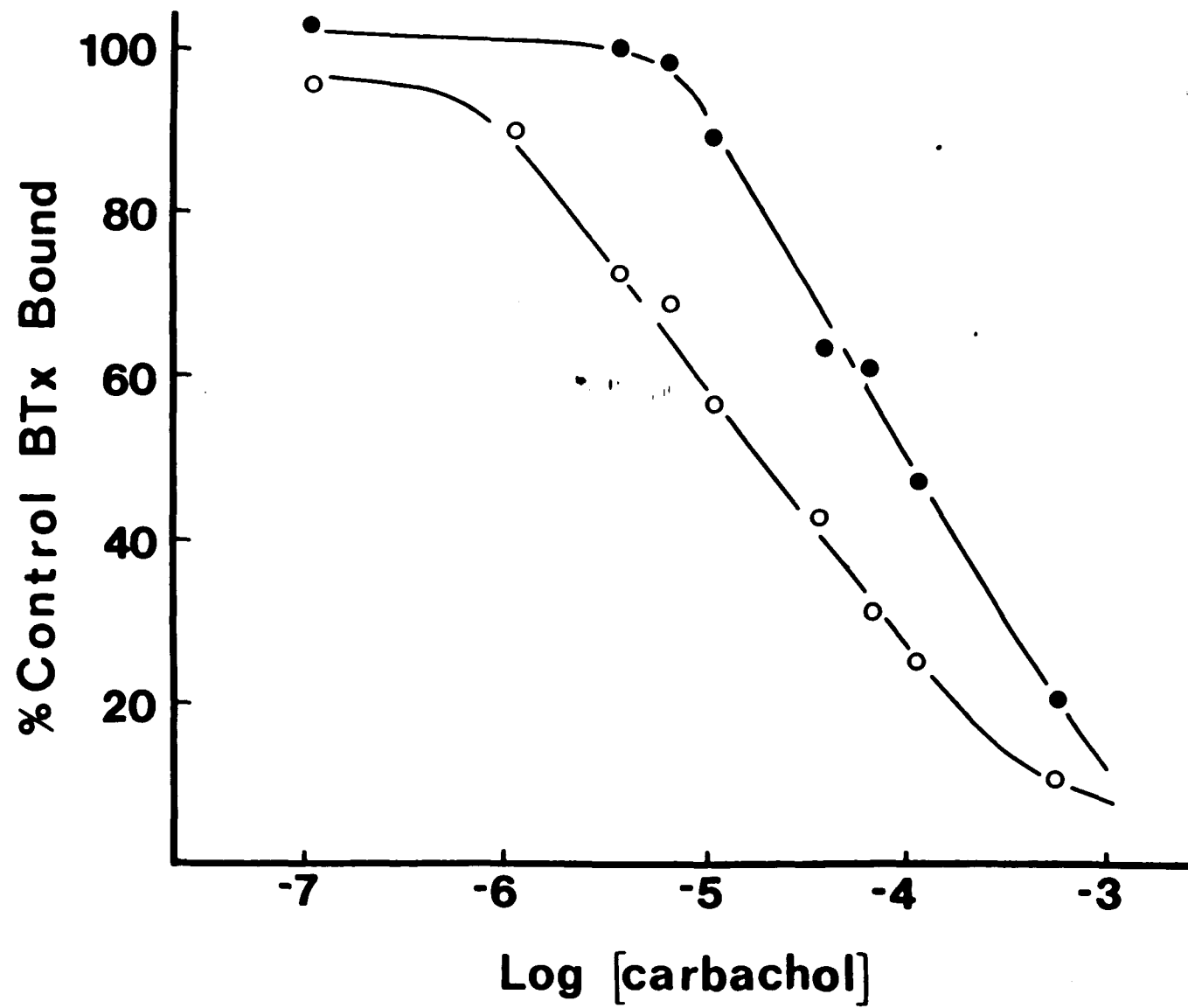
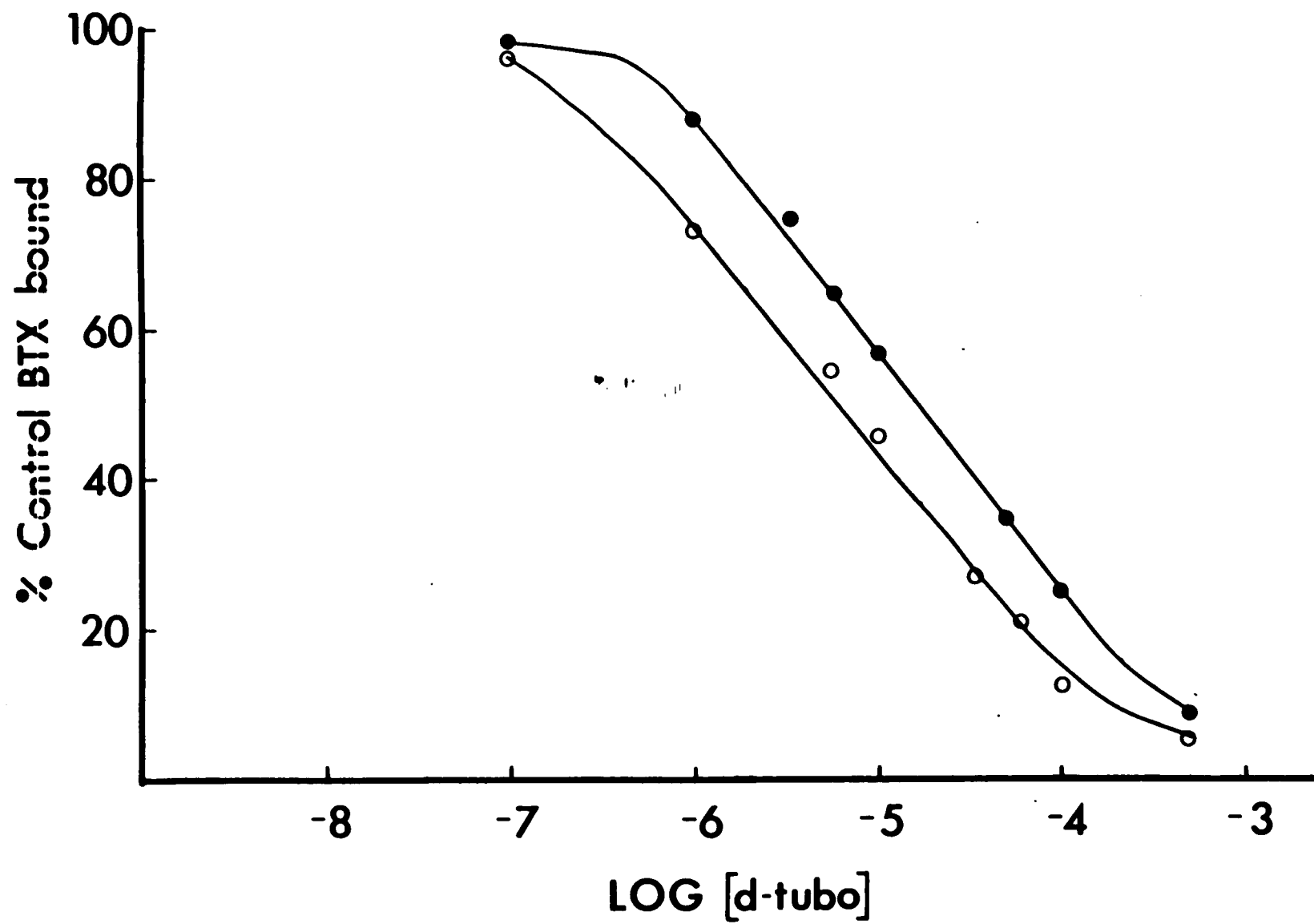


**Figure 36.** Competition by dTC, in the presence or absence of PCP, for  $\alpha$ -BTX binding to solubilized nAChR. Varying concentrations of dTC alone (open circles) or dTC in the presence of  $2 \times 10^{-8}$  M PCP (filled circles) were preincubated for 60 min at 4°C with Triton extracted sartorius nAChR. Conditions for the competition binding experiment and analysis were as described in figs. 34 and 35. The  $IC_{50}$  values for dTC inhibition of [ $^{125}$ I]  $\alpha$ -BTX binding were found to be 6.43  $\mu$ M (Hill coefficient=0.61) in the absence and 14.1  $\mu$ M (Hill coefficient=0.66) in the presence of PCP, respectively.



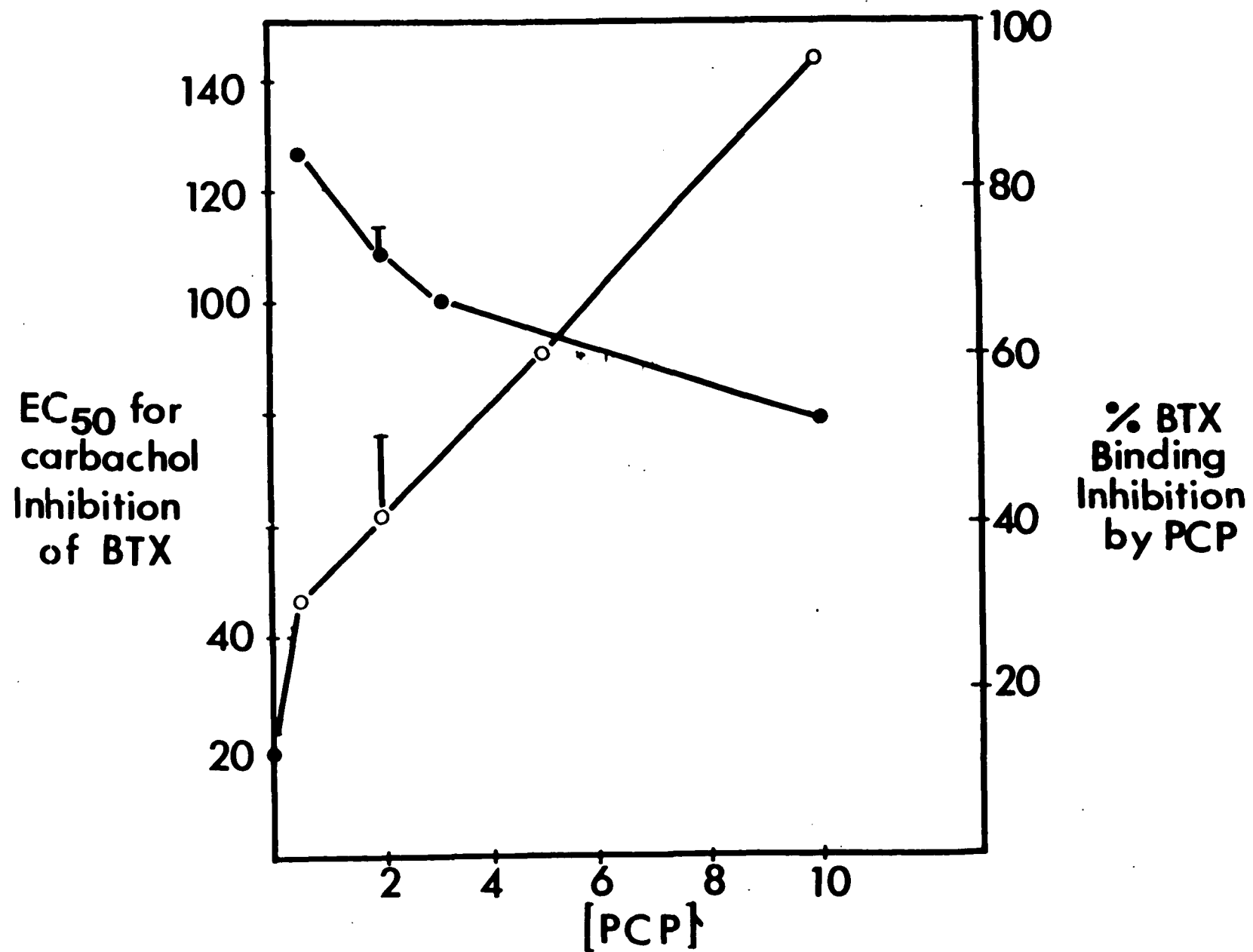
other ligand binding sites or possibly a selective elimination by PCP of one (higher affinity) of two classes of ligand binding sites. Similar results to those observed with carbachol, but to a lesser degree, were obtained with dTC. The  $IC_{50}$  for dTC inhibition of toxin binding increased from  $5.32 \pm 1.14 \mu M$  (Hill coefficient =  $0.63 \pm 0.02$ ) to  $11.6 \pm 3.6 \mu M$  (Hill coefficient =  $0.79 \pm 0.08$ ) in the presence of  $2 \times 10^{-6} M$  PCP, a 2.2-fold increase.

The effect of PCP to decrease the apparent affinity of carbachol as a competitor for toxin binding was found to be dose related (fig. 37). At a concentration of  $1 \mu M$  PCP, the  $IC_{50}$  for carbachol was determined to be  $143 \mu M$ , a 7.6-fold increase over the  $IC_{50}$  determined in the absence of PCP ( $18.9 \mu M$ ). This action of PCP may be mediated by an allosteric effect such that PCP directly alters the conformation of the carbachol binding site. An alternate possibility is that there exists a high and low affinity carbachol binding site for the inhibition of toxin binding. PCP may selectively inhibit the binding site which has the higher affinity for carbachol. If so, the remaining toxin binding sites are those that have a low affinity for carbachol. Consistent with the latter possibility is the observation that the PCP induced increase in the  $IC_{50}$  value for carbachol inhibition of toxin binding parallels the direct inhibition by PCP of total toxin binding (fig. 37).

19. Effect of muscle pretreatment on the ability of selected ligands to inhibit [ $^{125}I$ ]  $\alpha$ -BTX binding to muscle extracts.

Competition binding studies were performed under conditions identical to those previously described ( $5 nM$   $\alpha$ -BTX for 24 hrs at  $4^\circ C$ ), however, using muscle extracts prepared from DTT reduced and DPA alkylated whole sartorius muscle. Competition of dTC, carbachol and PCP for

**Figure 37.** Effect of PCP on the binding of  $\alpha$ -BTX and the ability of carbachol to compete for  $\alpha$ -BTX binding to solubilized nAChR. The  $IC_{50}$  for carbachol inhibition of toxin binding was determined as a function of the concentration of PCP with which it was coincubated (open circles). Protocol for  $IC_{50}$  determination was as described in figs. 34 and 35. All points represent the  $EC_{50}$  value for carbachol calculated from a single experiment with the exception of the 200 $\mu$ M PCP data point (n=4). Also shown is the direct effect of PCP as an inhibitor of toxin binding (filled circles). Each of these data points represents the mean of at least two individual experiments.



[<sup>125</sup>I] α-BTX binding was studied in each of these tissues for comparison with results obtained for these ligands in untreated muscle extracts. Results obtained from these studies are summarized in table 8. A significant increase (P<.01) of 15-fold is noted in the IC<sub>50</sub> value for carbachol inhibition of toxin binding in DPA alkylated muscle relative to that for control (untreated) muscle extracts. DTT treatment alone increases the IC<sub>50</sub> of carbachol about 4 fold. Similarly, significant increases over control are observed in the IC<sub>50</sub> values of dTC inhibition of toxin binding in DPA alkylated and DTT reduced muscle extracts of 3.8-fold and 2.2-fold, respectively. In contrast, the IC<sub>50</sub> values of PCP inhibition of toxin binding is not affected by either treatment. In each case, an IC<sub>50</sub> of about 1mM is observed.

20. Competition of ligands for toxin binding to untreated and DPA alkylated muscle extracts at 180 minutes.

In light of the results suggesting two classes of sites for α-BTX binding in muscle extracts, a study of the competition by ligands after a relatively short period of incubation would provide information about the high affinity (fast kinetics) as opposed to the low affinity (slow kinetics) population of toxin binding sites. Figure 38 shows the net toxin bound after 180 minutes of 5nM [<sup>125</sup>I] α-BTX association with muscle extracts as a function of concentration of carbachol, dTC or PCP. Results obtained for untreated and DPA alkylated muscle extracts indicate that dTC and PCP are approximately equieffective in each type of preparation as competitors for toxin binding (IC<sub>50</sub> approximately 8.5μM and 800μM, respectively). In contrast, a 2.3 fold increase is observed in the IC<sub>50</sub> for carbachol inhibition of toxin binding in DPA alkylated muscle relative to that observed in an untreated muscle

Table 8. Competition of ligands for BTX binding to soluble AChR in extracts prepared from untreated DTT reduced and DPA alkylated whole sartorius muscle.<sup>1</sup>

EC<sub>50</sub> value for inhibition of toxin binding<sup>1</sup>

|                            | carbachol (uM) | dTC (uM)    | PCP (uM) |
|----------------------------|----------------|-------------|----------|
| untreated                  | 18.9 ± 6.3     | 5.32 ± 1.14 | 1039     |
|                            | (n=4)          | (n=3)       | 1017     |
| DTT treated <sup>2</sup>   | 87             | 12.2        | 830      |
|                            | 62             | 10.0        | (n=1)    |
| DPA alkylated <sup>3</sup> | 296.7 ± 92.9   | 20.3 ± 5.5  | 780      |
|                            | (n=3)          | (n=3)       | 1080     |

1. Incubation of receptor with toxin (5nM) and ligand was carried out for 24 hours at 4°C.
2. DTT treatment was performed by incubation of whole sartorius muscle in 2mM DTT for 20 minutes at 23°C. Muscles were washed and extracted as described in the methods section.
3. DPA alkylation was performed by incubation of whole DTT treated sartorius muscles in 5 x 10<sup>-4</sup>M DPA for 30 minutes at 23°C. Muscles were then washed and extracted as described in the methods section.

**Figure 38.** Competition of carbachol, dTC and PCP for  $\alpha$ -BTX binding to extracts from untreated and DPA alkylated muscle (180 min). Samples of nAChR present in Triton X-100 extracts from sartorii that were untreated or DPA alkylated (see notes to table 8), were preincubated for 60 min at 4° C with the desired concentration of competing ligand. The binding reaction was begun by administration of [ $^{125}$ I]  $\alpha$ -BTX to give a final concentration of 5nM. After a 3 hr incubation at 4° C the toxin binding reaction was stopped by addition of a 50-fold excess of unlabelled  $\alpha$ -BTX. Duplicate aliquots from each sample were assayed by the Con A method for receptor-toxin complex. The IC<sub>50</sub> for inhibition of toxin binding was determined graphically from regression analysis of the linear portions of the dose response curves. Triangles, circles, and squares are used to designate carbachol, dTC and PCP competition, respectively. Solid symbols denote binding to DPA alkylated and hollow symbols to untreated muscle extracts. The IC<sub>50</sub> values for dTC and PCP were 8.5 $\mu$ M and 1050 $\mu$ M, respectively, in untreated muscle. Similar values were obtained with DPA alkylated muscle extracts (8.2 $\mu$ M and 1030 $\mu$ M, respectively). The IC<sub>50</sub> of carbachol, on the other hand, was significantly altered by DPA alkylation (20 $\mu$ M in untreated and 46 $\mu$ M in DPA alkylated extracts).

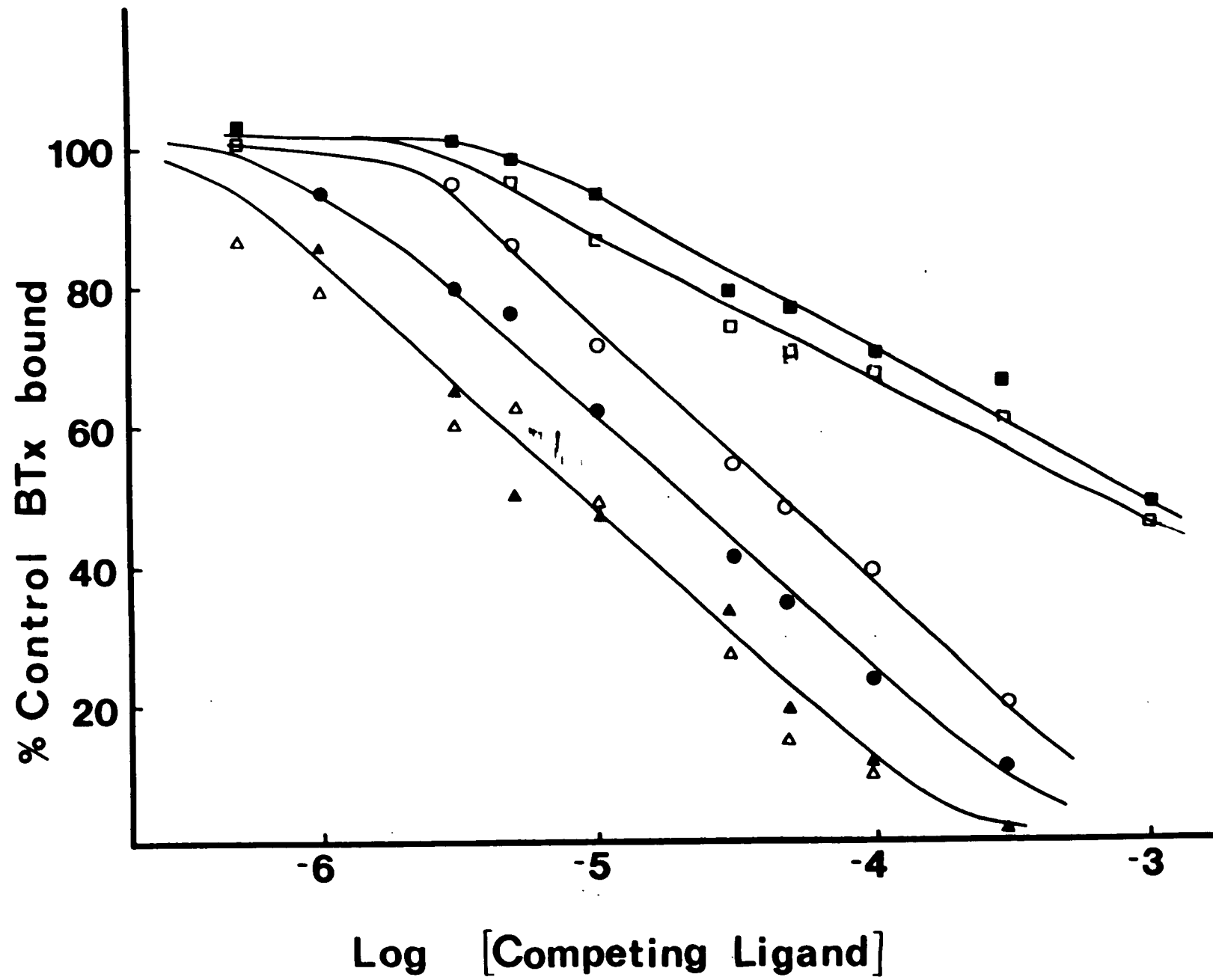


Table 9. 180 minute competition of ligands for BTX binding to solubilized AChR in extracts prepared from untreated and DPA alkylated whole sartorius muscle.

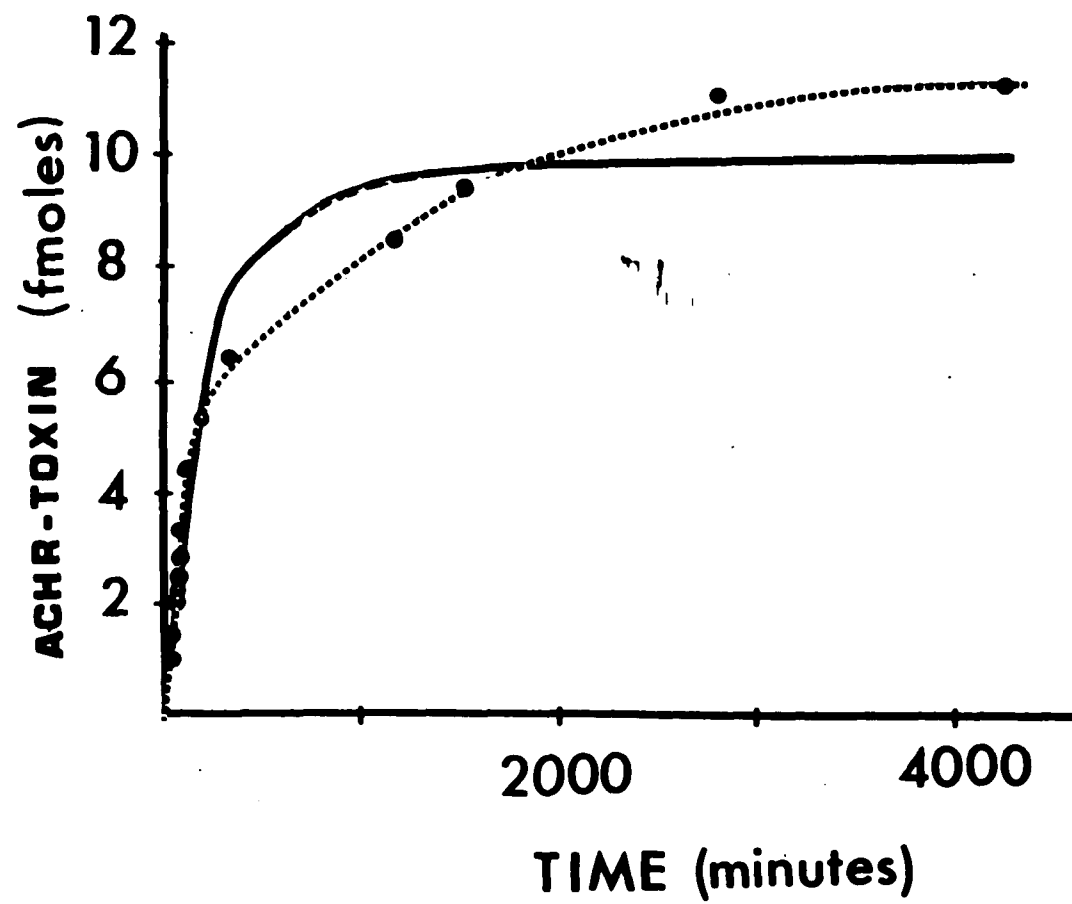
EC<sub>50</sub> value for toxin binding inhibition<sup>1</sup>

|                            | Carbachol (uM) | dTC (uM) | PCP (uM) |
|----------------------------|----------------|----------|----------|
| untreated                  | 20             | 8.5      | 1050     |
|                            | 12             | 8.0      | (n=1)    |
| DPA alkylated <sup>2</sup> | 45             | 6.0      | 1030     |
|                            | 30             | 7.5      | (n=1)    |

1. Incubation of receptor with toxin (5nM) and ligand was carried out at 4°C for 180 minutes. Binding reactions were stopped at this time by addition of a 50-fold excess of cold toxin.

2. DPA alkylation was performed as described in notes to table 8.

Figure 39. Kinetics of  $\alpha$ -BTX association to Triton X-100 solubilized receptors. The results of a typical experiment are shown. The binding reaction is initiated by the addition of labelled toxin at [3nM] and allowed to proceed at 4° C. At desired times the reaction is sampled by withdrawal of an aliquot and transferal to a vial containing a 100-fold excess of cold toxin (300nM). Each data point represents the mean of duplicate assays for toxin-receptor complex. The solid line is the computer determined best fit to a single kinetic component model, and the dashed line is the computer fit to a two component model. Values obtained for kinetic constants are  $17.0 \pm 2.5 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$  for a one component, and  $59.36 \pm 5.52 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$  and  $2.51 \pm .29 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$  for a two component system.



extract. The results of these competition experiments performed after 180 minute (short time) incubations are summarized in table 9 for comparison with data obtained after 24 hrs of toxin labelling (table 8). Little or no difference is observed in the ability of each of the three ligands to compete for toxin binding at either 3 or 24 hrs of toxin binding in untreated extracts, but differences are noted for DPA alkylated muscle extracts. The large increase in  $IC_{50}$  for carbachol and dTC observed after DPA alkylation of muscle following a 24 hr toxin incubation is either greatly reduced or absent, respectively, after the short 3 hr incubation with toxin. These findings suggest that DPA alkylation differentially affects the fast and slow sub-populations of  $\alpha$ -BTX binding sites.

21. Kinetics of [ $^{125}$ I]  $\alpha$ -BTX binding to untreated, DTT reduced and DPA alkylated sartorius muscle extracts.

In order to obtain more information regarding the nature of [ $^{125}$ I]  $\alpha$ -BTX binding sites in detergent solubilized receptor preparations, a detailed analysis of the rate of toxin association was carried out. The results of a typical experiment are shown in fig. 39. Total  $\alpha$ -BTX bound was determined at selected intervals between 5 minutes and 4227 minutes. The experimental data have been analyzed, assuming a second order rate process for binding, to determine whether they can be explained by a model involving a single kinetic component or whether the results require postulation of two or more kinetic components. Analyses were performed using the Prophet computer curve fitting and non-linear regression programs applied to the theoretical rate equations for a one component and a two component system (described in the Materials and Methods section). The solid line in figure 39 is the computer derived fit to a

single component model, and the dashed line represents the two component model. Table 10 summarizes results obtained for the evaluation of kinetic constant(s) and number of  $\alpha$ -BTX binding sites available in untreated, DTT treated, and DPA alkylated muscle evaluated for both a one and a two kinetic component system. An analysis of the variance in the data points between that experimentally determined and the theoretical result based on the alternative models is shown in the table as the sum of squares ( $\pm$ S.E.M.) and the residual ( $\pm$ S.E.M.). The model with the smaller residual is considered to provide a better description of the data. By this criteria the two kinetic component model, which showed significantly lower residuals than the one component model in all three types of muscle extracts ( $P < .01$ ), is accepted as the one which provides the best description of the data.

Allowing the computer to simultaneously optimize for each of the four unknowns in the binding rate equation ( $R_1$ ,  $R_2$ ,  $K_1$  and  $K_2$ ); (see data analysis section of Materials and Methods) for a two component model, results in the estimation of a pair of rate constants for each binding curve. These best fit constants are found to be closely similar in untreated, DTT reduced, and DPA alkylated muscle extracts (table 10). The two derived rate constants vary by a factor of 20, the faster one being  $50 \times 10^{-5} \text{ M}^{-1} \text{ min}^{-1}$  and the slower one,  $2.5 \times 10^{-5} \text{ M}^{-1} \text{ min}^{-1}$ . Each of the two kinetic components represents roughly 50% of the total number of  $\alpha$ -BTX binding sites in untreated muscle. However, DTT reduction of the muscle results in a significant decrease in the total number of  $\alpha$ -BTX binding sites to 74.9% of control, ( $P < .05$ ). Reduction followed by alkylation with DPA results in a further decrease to 63.0% of control,  $P < .01$ . Although some apparent decrease in concentration is

Table 10. Kinetic analyses of [ $^{125}$ I]  $\alpha$ -BTX association to soluble nAChR in sartorius muscle extracts.

|                                                          | untreated<br>(n=3) | DTT reduced<br>(n=3) | DPA alkylated<br>(n=3) |
|----------------------------------------------------------|--------------------|----------------------|------------------------|
| Two site model:                                          |                    |                      |                        |
| $R_1$ (fmoles/muscle)                                    | $94.98 \pm 23.12$  | $56.0 \pm 9.44$      | $44.20 \pm 5.76$       |
| $R_2$ (fmoles/muscle)                                    | $82.0 \pm 22.8$    | $76.36 \pm 10.02$    | $67.20 \pm 7.80$       |
| $R_{\text{total}}$ (fmoles/muscle)                       | $177.00 \pm 12.20$ | $132.5 \pm 19.48$    | $111.56 \pm 16.72$     |
| $K_1$ ( $\times 10^5 \text{M}^{-1} \text{min}^{-1}$ )    | $50.1 \pm 8.1$     | $55.9 \pm 18.1$      | $51.3 \pm 1.8$         |
| $K_2$ ( $\times 10^{-5} \text{M}^{-1} \text{min}^{-1}$ ) | $2.58 \pm .06$     | $2.52 \pm .64$       | $2.82 \pm .77$         |
| S.S. (sum of squares)                                    | $189.3 \pm 4.8$    | $103.7 \pm 21.4$     | $80.1 \pm 24.2$        |
| Residual                                                 | $0.708 \pm .29$    | $0.51 \pm .11$       | $0.41 \pm .01$         |
| One site model:                                          |                    |                      |                        |
| $R_{\text{total}}$ (fmoles/muscle)                       | $151.76 \pm 12.08$ | $107.6 \pm 14.54$    | $93.2 \pm 11.20$       |
| $K_1$ ( $\times 10^5 \text{M}^{-1} \text{min}^{-1}$ )    | $22.2 \pm 7.1$     | $18.5 \pm 4.6$       | $16.2 \pm 5.3$         |
| S.S.                                                     | $156.19 \pm 41.2$  | $96.8 \pm 17.4$      | $76.1 \pm 22.5$        |
| Residual                                                 | $10.16 \pm 3.96$   | $7.37 \pm 4.27$      | $4.32 \pm 1.73$        |

observed for each of the kinetically distinct sub-populations of toxin binding sites, it is only the fast component that is significantly reduced as a result of either reduction or alkylation ( $p < .01$  for both treatment groups).

22. One site vs. two site system: method of distribution of residuals.

One would expect that the greater the number of kinetic components allowed for a computer fit to a given set of experimental data (i.e., the greater the number of variables) the better will be the fit. For this reason, the distinction between a model involving a single vs. two kinetic components by assessment of the residual variation in the data not accounted for by the model is not entirely satisfactory. A frequency distribution of the difference between the theoretical value (predicted by a one site or two site model) and the experimentally measured value for toxin bound at each time point in a given experiment provides a better evaluation of the goodness of fit. If a given model is correct it is predicted that this frequency distribution will be centered around zero. Alternatively, an incorrect model should exhibit some skewness to the frequency distribution. Table 11 displays the mean of the peaks of frequency distributions for the residual variation in all the data points of 20 individual kinetic experiments. When the data were evaluated for a single rate constant for toxin binding the mean peak frequency distribution is significantly different from zero ( $.452 \pm .188$ ,  $n=20$ ). In the two site model, the mean peak frequency distribution is not significantly different from zero ( $0.04 \pm 0.08$ ;  $n=20$ ). It is therefore concluded that a two kinetic site model, which includes zero in the mean of the peaks for frequency distributions of residual variation ( $.04 \pm .086$ ,  $n=20$ ), is the simplest model with adequate fit to the data.

Table 11. Frequency distribution of residuals calculated for a one component and a two component kinetic model for the kinetics of toxin association.

|           | Mean of frequency distribution<br>peaks ( $\pm$ S.E.M.) | N  |
|-----------|---------------------------------------------------------|----|
| one site: | $0.452 \pm 0.186$                                       | 20 |
| two site: | $0.046 \pm 0.086$                                       | 20 |

23. Effect of carbachol on the kinetics and number of [ $^{125}$ I]  $\alpha$ -BTX binding in sartorius muscle extracts.

Computer evaluation of the kinetics of  $\alpha$ -BTX binding in the presence of  $10^{-8}$  M carbachol indicates no significant reduction in the rate constants  $K_1$  and  $K_2$  relative to control for untreated, DTT reduced, and DPA alkylated muscle extracts (see table 12). However, significant decreases in the number of fast toxin binding sites, but not the slower kinetic component are observed in each of these types of pretreated tissues ( $p < .01$ ). The total reduction in the number of fast toxin binding sites measured in the presence of  $10^{-8}$  M carbachol is no different whether the extract is derived from reduced, alkylated or untreated muscle (although DTT reduction and DPA alkylation alone result in a diminution in the fast component-see table 9). A dose dependency is observed for carbachol inhibition of the fast toxin binding component. Table 13 displays the results obtained for the number and kinetic rate constants of [ $^{125}$ I]  $\alpha$ -BTX binding sites for untreated muscle extract in the presence of three concentrations of carbachol. While there is a consistent decrease in the amount of the fast toxin binding sites with increasing carbachol concentration, the presence of agonist does not affect the amount of the slow toxin binding sites present nor the magnitude of either of the rate constants. At the highest concentration of carbachol used ( $75\mu\text{M}$ ) the fast toxin binding component is not measurable with any accuracy; the estimated parameters are unrealistic, i.e.,  $R_1$  is negative and  $k_1$  is not different from zero,  $10^3 \pm 10^3$ . Such estimates would be obtained if the fast component were eliminated by this concentration of carbachol. The lack of effect of lower concentrations of carbachol on either of the kinetic rate

Table 12. Effect of  $10^{-5}$  M carbachol on  $[^{125}\text{I}] \alpha\text{-BTX}$  association kinetics and on number of sites labelled in soluble AChR from untreated DTT reduced and DPA alkylated sartorius muscle extract.

|                                                       | untreated (n=3)  |                  | DTT reduced (n=3) |                 | DPA alkylated (n=3) |                 |     |
|-------------------------------------------------------|------------------|------------------|-------------------|-----------------|---------------------|-----------------|-----|
|                                                       | control          | carbachol        | control           | carbachol       | control             | carbachol       |     |
| $R_1$ (fmoles/muscle)                                 | $95.0 \pm 23.1$  | $24.9 \pm 1.4$   | $56.0 \pm 9.4$    | $20.6 \pm 4.9$  | $44.7 \pm 5.7$      | $15.0 \pm 10.8$ |     |
| $R_2$ (fmoles/muscle)                                 | $82.0 \pm 22.8$  | $89.0 \pm 24.8$  | $76.5 \pm 10.0$   | $69.4 \pm 4.1$  | $67.2 \pm 7.8$      | $65.4 \pm 20.9$ |     |
| $R_T$ (fmoles/muscle)                                 | $177.0 \pm 12.2$ | $113.9 \pm 27.6$ | $132.5 \pm 19.5$  | $90.0 \pm 13.4$ | $111.4 \pm 16.7$    | $80.4 \pm 31.6$ | 177 |
| $K_1$ ( $\times 10^5 \text{M}^{-1} \text{min}^{-1}$ ) | $50.1 \pm 8.1$   | $66.4 \pm 9.5$   | $55.9 \pm 18.1$   | $59.1 \pm 7.9$  | $51.3 \pm 1.8$      | $40.0 \pm 1.9$  |     |
| $K_2$ ( $\times 10^5 \text{M}^{-1} \text{min}^{-1}$ ) | $2.58 \pm 0.06$  | $3.55 \pm 1.07$  | $2.52 \pm 0.64$   | $2.92 \pm 1.33$ | $2.82 \pm 0.77$     | $1.94 \pm 0.55$ |     |

Table 13. Effect of different carbachol concentrations on the association kinetics of  $[^{125}\text{I}]$   $\alpha$ -BTX and the number of sites in extracts of untreated sartorius muscle.

|                                                       | CARBACHOL          |                                     |                                       |                                       |
|-------------------------------------------------------|--------------------|-------------------------------------|---------------------------------------|---------------------------------------|
|                                                       | 0<br>(n=3)         | $4 \times 10^{-6}\text{M}$<br>(n=1) | $2.5 \times 10^{-5}\text{M}$<br>(n=1) | $7.5 \times 10^{-5}\text{M}$<br>(n=1) |
| $R_1$ (fmoles/muscle)                                 | $94.98 \pm 23.12$  | $28.46 \pm 7.48$                    | $21.96 \pm 11.32$                     | -1.77                                 |
| $R_2$ (fmoles/muscle)                                 | $82.00 \pm 22.80$  | $96.60 \pm 6.34$                    | $88.76 \pm 10.7$                      | $95.58 \pm 8.6$                       |
| $R_T$ (fmoles/muscle)                                 | $176.98 \pm 12.20$ | $125.06 \pm 0.91$                   | $110.72 \pm 11.01$                    | 93.81                                 |
| $K_1$ ( $\times 10^5 \text{M}^{-1} \text{min}^{-1}$ ) | $50.1 \pm 8.1$     | $64.6 \pm 24.0$                     | $54.8 \pm 1.17$                       | $10^5 \pm 1 \times 10^8$              |
| $K_2$ ( $\times 10^5 \text{M}^{-1} \text{min}^{-1}$ ) | $2.58 \pm .06$     | $3.53 \pm .10$                      | $2.22 \pm 1.14$                       | $2.53 \pm .59$                        |

constants for toxin binding rules out a simple competition between the two ligands for binding to a common site.

24. Effect of dTC on the kinetics and number of [ $^{125}$ I]  $\alpha$ -BTX binding sites in detergent extracted sartorius muscle.

Competition by  $10^{-5}$  M dTC for  $\alpha$ -BTX binding to untreated muscle extract affects neither the fast nor the slow rate constant derived for the binding reactions (see table 14). However, in common with carbachol, dTC causes a significant reduction in the total number of fast binding sites (but not slow binding sites) in each of the three treatment groups ( $P < .01$ ). The dTC inhibition is the same for all groups since the computer derived concentration for the fast toxin binding component, in the presence of  $10^{-5}$  M dTC, is not significantly different in untreated, DTT reduced and DPA alkylated muscle extracts. Increasing the concentration of dTC results in a progressive decrease in the number of fast toxin binding sites (table 15). At the highest concentration of dTC studied (25  $\mu$ M) only 6.7% of the total toxin sites are of the fast kinetic component compared to  $55.3 \pm 10.6\%$  in the absence of dTC. However, at this concentration dTC does not alter either the number of slower toxin binding sites or the magnitude of either of the kinetic constants. Previous results have demonstrated that greater than 90% of the  $\alpha$ -BTX bound after 24 hrs can be eliminated by coincubation with millimolar concentrations of dTC or carbachol. Thus higher concentration of competitor probably can produce an inhibition of the slow toxin binding site. However, these concentrations of dTC and carbachol were not evaluated in kinetic experiments.

Table 14. Effect of  $10^{-5}M$  dTC on  $[^{125}I]\alpha$ -BTX association kinetics and number of sites in extracts from untreated, DTT reduced, and DPA alkylated sartorius muscles.

|                                         | untreated        |                    | DTT reduced      |                    | DPA alkylated    |                    |
|-----------------------------------------|------------------|--------------------|------------------|--------------------|------------------|--------------------|
|                                         | control          | dTC ( $10^{-5}M$ ) | control          | dTC ( $10^{-5}M$ ) | control          | dTC ( $10^{-5}M$ ) |
| $R_1$ (fmoles/muscle)                   | $95.0 \pm 23.1$  | $29.6 \pm 3.9$     | $56.0 \pm 9.1$   | $22.9 \pm 1.2$     | $44.2 \pm 5.7$   | $27.9 \pm 5.5$     |
| $R_2$ (fmoles/muscle)                   | $82.0 \pm 22.8$  | $72.0 \pm 2.9$     | $76.5 \pm 10.0$  | $62.0 \pm .40$     | $67.2 \pm 7.8$   | $54.4 \pm 17.7$    |
| $R_T$ (fmoles/muscle)                   | $177.0 \pm 12.2$ | $101.6 \pm 10.4$   | $132.5 \pm 19.5$ | $84.9 \pm .51$     | $111.4 \pm 16.7$ | $82.3 \pm 23.8$    |
| $K_1$ ( $\times 10^5 M^{-1} min^{-1}$ ) | $50.1 \pm 8.1$   | $40.1 \pm 15.0$    | $55.9 \pm 18.1$  | $36.3 \pm 6.8$     | $51.3 \pm 18.0$  | $35.7 \pm 3.07$    |
| $K_2$ ( $\times 10^5 M^{-1} min^{-1}$ ) | $2.58 \pm 0.06$  | $5.00 \pm 1.56$    | $2.52 \pm 0.64$  | $3.45 \pm 0.50$    | $2.82 \pm 0.77$  | $2.12 \pm 0.84$    |

Table 15. Dose effect of dTC on  $[^{125}\text{I}]\alpha\text{-BTX}$  association kinetics and number of sites in extract of untreated sartorius muscle.

|                                                       | dTC                |                                     |                                     |                                       |
|-------------------------------------------------------|--------------------|-------------------------------------|-------------------------------------|---------------------------------------|
|                                                       | 0<br>(n=3)         | $4 \times 10^{-6}\text{M}$<br>(n=1) | $1 \times 10^{-5}\text{M}$<br>(n=3) | $2.5 \times 10^{-5}\text{M}$<br>(n=1) |
| $R_1$ (fmoles/muscle)                                 | $94.98 \pm 23.12$  | $51.52 \pm 4.66$                    | $29.60 \pm 5.72$                    | $6.16 \pm 2.90$                       |
| $R_2$ (fmoles/muscle)                                 | $82.00 \pm 22.80$  | $77.4 \pm 10.18$                    | $71.98 \pm 4.88$                    | $85.70 \pm 3.52$                      |
| $R_T$ (fmoles/muscle)                                 | $176.98 \pm 12.20$ | $128.9 \pm 7.42$                    | $101.58 \pm 10.40$                  | $91.86 \pm 2.71$                      |
| $K_1$ ( $\times 10^5 \text{M}^{-1} \text{min}^{-1}$ ) | $50.1 \pm 8.1$     | $30.7 \pm 5.2$                      | $40.1 \pm 15.0$                     | $88.5 \pm 98.7$                       |
| $K_2$ ( $\times 10^5 \text{M}^{-1} \text{min}^{-1}$ ) | $2.50 \pm 0.06$    | $2.00 \pm 0.36$                     | $5.00 \pm 1.56$                     | $2.61 \pm 0.33$                       |

25. Effect of PCP on the kinetics and number of [ $^{125}$ I]  $\alpha$ -BTX binding sites in detergent extracted muscle.

PCP at  $2 \times 10^{-6}$  M does not significantly alter either the rate constants for the toxin binding reaction, or the total number of either class of sites (see table 16). This result is obtained for DTT reduced and DPA alkylated as well as untreated muscle extracts. Higher concentrations of PCP significantly decrease the total number of toxin binding sites (table 17). As was shown to be the case for both carbachol and dTC (at the concentrations studied) the decrease in total toxin binding sites is accounted for by a loss of the fast kinetic component. PCP at 0.5mM and 1.0mM produces a 44% and 60% reduction of the fast site respectively; while the concentration of the slow binding site is unchanged. A small (but not statistically significant) reduction in both rate constants for toxin binding is noted.

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Table 16. Effect of  $2 \times 10^{-4}$  M PCP on  $[^{125}\text{I}]\alpha\text{-BTX}$  association kinetics and number of sites in extracts of untreated, DTT reduced, and DPA alkylated muscles.

|                                                       | untreated        |                  | DTT reduced      |                 | DPA alkylated    |                 |
|-------------------------------------------------------|------------------|------------------|------------------|-----------------|------------------|-----------------|
|                                                       | control          | PCP              | control          | PCP             | control          | PCP             |
| $R_1$ (fmoles/muscle)                                 | 95.0 $\pm$ 23.1  | 88.6 $\pm$ 2.6   | 56.0 $\pm$ 9.1   | 90.3 $\pm$ 17.0 | 44.2 $\pm$ 0.51  | 43.8 $\pm$ 3.3  |
| $R_2$ (fmoles/muscle)                                 | 82.0 $\pm$ 22.8  | 103.1 $\pm$ 17.1 | 76.5 $\pm$ 10.0  | 72.7 $\pm$ 15.8 | 67.2 $\pm$ 7.8   | 66.0 $\pm$ 6.5  |
| $R_T$ (fmoles/muscle)                                 | 177.0 $\pm$ 12.2 | 191.6 $\pm$ 17.1 | 132.5 $\pm$ 19.5 | 163.0 $\pm$ 2.7 | 111.4 $\pm$ 16.7 | 99.8 $\pm$ 10.0 |
| $K_1$ ( $\times 10^5 \text{M}^{-1} \text{min}^{-1}$ ) | 50.1 $\pm$ 8.1   | 45.2 $\pm$ 7.4   | 55.9 $\pm$ 18.1  | 39.6 $\pm$ 7.2  | 51.3 $\pm$ 1.8   | 34.6 $\pm$ 8.0  |
| $K_2$ ( $\times 10^5 \text{M}^{-1} \text{min}^{-1}$ ) | 2.58 $\pm$ 0.06  | 2.98 $\pm$ 1.27  | 2.52 $\pm$ 0.64  | 2.61 $\pm$ 0.44 | 2.82 $\pm$ 0.77  | 1.52 $\pm$ 0.25 |

Table 17. Dose effect of PCP on  $[^{125}\text{I}]\alpha\text{-BTX}$  association kinetics and number of sites in extracts of untreated sartorius muscles.

|                                                      | PCP              |                            |                            |                  |
|------------------------------------------------------|------------------|----------------------------|----------------------------|------------------|
|                                                      | 0                | $2 \times 10^{-4}\text{M}$ | $5 \times 10^{-4}\text{M}$ | $1\text{mM}$     |
|                                                      | (n=3)            | (n=3)                      | (n=1)                      | (n=1)            |
| $R_1$ (fmoles/muscle)                                | $95.0 \pm 25.1$  | $88.6 \pm 2.6$             | $51.7 \pm 5.3$             | $38.6 \pm 10.8$  |
| $R_2$ (fmoles/muscle)                                | $82.0 \pm 22.8$  | $103.1 \pm 17.1$           | $71.4 \pm 4.8$             | $76.9 \pm 9.7$   |
| $R_T$ (fmoles/muscle)                                | $177.0 \pm 12.2$ | $191.6 \pm 17.1$           | $123.1 \pm 10.2$           | $115.5 \pm 20.3$ |
| $K_1$ ( $\times 10^5 \text{M}^{-1}\text{min}^{-1}$ ) | $50.1 \pm 8.1$   | $45.2 \pm 7.4$             | $32.1 \pm 5.2$             | $31.5 \pm 13.8$  |
| $K_2$ ( $\times 10^5 \text{M}^{-1}\text{min}^{-1}$ ) | $2.58 \pm 0.06$  | $2.40 \pm 1.27$            | $1.95 \pm 0.43$            | $1.95 \pm 0.81$  |

## DISCUSSION

Rapid and complete formation of the DPA ion from its 2-chloroethylamine precursor has been demonstrated. Consistent with its structural analogy to the phenyltrimethylammonium ion, DPA acts as an agonist on the frog rectus abdominis preparation ( $EC_{50}=17.0\mu M$ ; figure 3). The maximal response produced by DPA is  $91.9\pm 2.7\%$  of the maximum obtained by carbachol. The small deviation from full agonism may be the result of an antagonism or desensitization by the alcoholic product of the hydrolysis of the aziridinium ion (fig. 1). The nicotinic nature of DPA agonism on rectus muscle is supported by its blockade by low doses of (1) the nicotinic receptor antagonists,  $\alpha$ -BTX ( $130nM$ ) and curare ( $100\mu M$ ), and (2) the nicotinic channel blocking drugs, PCP ( $5\mu M$ ) and adiphenine ( $10\mu M$ ). Observation by Hunt (1948) of an acute flaccid paralysis accompanied by skeletal muscle fasciculations following intravenous administration of DPA in cats is consistent with nicotinic agonist activity.

Reduction of the disulphide bond(s) that has been established to be in close proximity or part of the agonist binding site on the nAChR at the neuromuscular junction results in modified responses to cholinergic agonists. A 3.67-fold increase in the  $EC_{50}$  for carbachol was observed after treatment of the frog rectus with DTT (figure 2). These findings are consistent with the 3.9- and 5.7-fold increases in  $EC_{50}$  for carbachol previously observed on the same tissue by Bleehan et al. (1980) and Fleisch et al. (1974), respectively. In conjunction with observations of a decrease in  $EC_{50}$  for decamethonium (1.66-fold) following DTT reduction of the rectus (Bleehan et al., 1980), an alteration in the conformation of the agonist binding site has been proposed as the mechanism for these changes in the ligand affinities.

The initial tension response to submaximal concentrations of DPA is diminished by prior reduction of the muscle with DTT (figure 4). However, in contrast to untreated muscle, only a partial reversal of the DPA induced response is obtained even after extensive drug washout of reduced muscle. The component of the response to DPA obtained in reduced muscle that is not reversed by washout is extremely stable and presumably is the result of alkylation of a sulfhydryl group thought to reside within a few angstroms of the agonist binding site on the nAChR of neuromuscular junction (Ben-Haim et al., 1973). Although there may be some ambiguity concerning the site of alkylation for an irreversibly acting cholinergic antagonist, i.e., it may alkylate at the active site on the receptor or at any other location which interferes with the receptor mediated ion conductance response; the results of these experiments suggest only the former is important for an irreversible agonist such as DPA.

Complete protection against the "irreversible" response resulting from DPA alkylation of reduced muscle was observed with all three classes of cholinergic ligand studied (agonist, antagonist and ion channel blocker; figures 5-7). Although it is not surprising that carbachol and dTC may afford protection by a direct competition for the binding site of DPA, protection by a channel blocker such as 25 $\mu$ M PCP (under conditions where it does not directly influence  $\alpha$ -bungarotoxin binding to the receptor (see binding experiments below) requires an alternate explanation. Protection can be the result not only of a direct competition for the binding of DPA at the same site but may also result from an indirect effect either on the conformation of the agonist binding site (such that DPA does not bind) or on the proximity of the

sulfhydryl group to the agonist binding site (such that DPA may bind but not alkylate). An indirect conformational effect on the channel receptor complex is suggested to account for the observed protection by PCP against the irreversible action of DPA on the reduced rectus muscle.

Reversal of the sustained response to DPA was produced by PCP, dTC, and  $\alpha$ -BTX (figures 8-10); all at concentrations in the range of their known antagonistic effects. Assuming the sustained action of DPA results from the formation of a covalent bond at the receptor site, reversion of the response by antagonists necessitates the simultaneous occupancy by both DPA and the blocking ligand on the receptor-channel complex. Simultaneous binding of PCP to the open conductance channel of the DPA bound activated conformation of the receptor can explain satisfactorily the reverting action of PCP. However, questions may be raised about the mechanisms of reversal by dTC and  $\alpha$ -BTX. The results of electrophysiological studies indicate that dTC behaves as an ion channel blocker at concentrations similar to those observed for its antinicotinic action at the neuromuscular junction (Steinbach et al., 1980; Eldefrawi et al. 1979). Thus, a channel blocking action of dTC can be proposed as the basis for the reversal of the "irreversible" DPA contracture as opposed to the alternative explanation that agonists and antagonists may bind simultaneously to distinct sites on the receptor itself. In the case of  $\alpha$ -BTX, where no direct channel blocking action has been reported, a receptor mechanism can be proposed. Since  $\alpha$ -BTX is a large molecule (8,000 daltons) which exhibits very high affinity for the receptor (nanomolar) binding by multipoint attachment seems likely. Although DPA alkylation may serve to diminish the affinity for toxin binding by obscuring one or more possible attachment sites, it may

be expected that at higher concentrations of toxin, occupancy of the receptor (along with inhibition of response) will occur due to attachment at the remaining sites. Thus toxin binding to nAChR closes channels in spite of the DPA bound to the receptor. This possibility is consistent with binding experiments (discussed below) which give direct evidence for the binding of toxin to DPA alkylated muscle. Although the number of toxin binding sites per intact muscle is observed to be identical in both DPA alkylated and untreated muscle, the apparent affinity for toxin binding is about 10-fold less in the former.

A complex anticholinergic action has been demonstrated for PCP at the neuromuscular junction depending on the doses used (figure 11). In agreement with observation by Maayani et al. (1974) a parallel shift in the cumulative log dose response curve for a cholinergic agonist was produced by  $1\mu\text{M}$  PCP on the frog rectus abdominis muscle preparation. Although it was noted by these researchers that higher doses of PCP result in a depression of the maximum response, data analysis assuming a competitive inhibition at lower doses yielded a  $K_I$  approximately equal to that obtained for dTC on this tissue ( $2 \times 10^{-7} \text{M}$ ). We have found that  $5\mu\text{M}$  PCP produces a greater than 50% reduction in the maximal response induced by carbachol, indicative of a non-competitive mode of antagonism. A maximal direct response can still be obtained with 30mM KCl indicating no effect of PCP on the contractile apparatus of the muscle. These findings are in accord with current evidence suggesting a channel blocking action of PCP on nicotinic channels (Albuquerque et al., 1979; Tsai et al., 1979) and a lack of effect on the recognition sites of the receptor itself (Kloog et al., 1979; Tsai et al., 1977; Mittag and Gross, 1979). A channel blocking action is also suggested by

the reversal of the sustained contracture induced by alkylation of the receptor with DPA. In common with PCP, other agents that have been reported to block the ion conductance channel associated with the nicotinic receptor such as amantidine (Tsai et al., 1978) and adifenine (Tiedt et al., 1978) also reverse DPA induced contracture (results not shown).

The basis for the apparent competitive antagonism of the carb response with low concentrations of PCP ( $1\mu\text{M}$ ) may be the result of a spare receptor and channel population, i.e., an excess over the number of receptors necessary for a maximal response by the tissue. Spare receptors have been reported to occur at cat (Paton and Waud, 1967) and mouse neuromuscular junctions (Barnard et al., 1971). The presence of spare receptors would allow for a full agonist response in the presence of a non-competitive antagonist (assuming less than the number of spare receptors were occupied by the antagonist) by the dose-dependent increase in the fraction of unblocked receptors occupied by agonist. Under these conditions a maximal response will be obtained, but a depression in the slope of the log dose response curve is expected. Consistent with this prediction, at antagonist concentrations less than that necessary to depress the maximal response, the slope of the log dose response curve for carbachol was found to decrease from  $1.92 \pm .23$  in control muscle to  $1.34 \pm .18$  in the presence of  $1\mu\text{M}$  PCP.

An agent which binds to the nicotinic ion channel may be expected to differentiate between channels in the conducting (open) and nonconducting (closed) conformations, since these states probably represent major differences in conformation. Although PCP clearly has an affinity for the closed channels associated with the resting conformation of

receptor, the experimental results obtained suggest that it has a higher affinity for the agonist activated-open channel. Sequential carbachol dose response curves generated from muscles equilibrated with 5 $\mu$ M PCP (allowing a 1 hr "rest," equal to re-equilibration and recovery from agonist exposure, between agonist doses, in the continued presence of PCP) indicate no depression of the maximum response, only a shift to the right of the dose response curve (figure 14). This contrasts with a greater than 50% depression of the maximum response observed in the presence of the identical concentration of PCP when the dose of carbachol is varied cumulatively. An enhanced affinity of PCP for the agonist activated conformation of the channel which is present in the sustained presence of agonist but not during the shorter exposure used in the sequential method of constructing carbachol dose-response curves may account for this disparity. In support of this interpretation experiments involving a fixed schedule of carbachol addition and washout yield a progressive decline of response to a plateau level in muscle continuously equilibrated with PCP (figure 16). This is in contrast to the uniform response obtained with sequential agonist doses in control muscle and sequential or cumulative carbachol in the presence of adiphenine. These results suggest that the original equilibrium established between PCP and its binding site (the closed ion channels) is perturbed by the presence of agonist. The fade in response to successive agonist challenges is believed to result from the enhanced affinity of PCP for open relative to closed channels which does not dissociate from channels during the agonist washout portion of the short time cycle. When the cycle is extended to 1 hr., this effect is not seen. A PCP concentration dependence is noted for both the initial

response as well as the plateau level of response obtained after a series of agonist challenges (figure 19). It is proposed that the initial response approximates the extent of inhibition due to PCP binding to closed channels (there may be a component of open channel binding due to the need for agonist to make the measurement) whereas the plateau response approximates to the inhibition resulting from PCP binding to agonist activated channels. If this interpretation is correct, analysis of the dose response relationships indicates a greater than 2-fold affinity for open relative to closed channels. These findings are in agreement with a report that the affinity of PCP binding to nicotinic channels in electroplax membranes increases from a  $K_d$  value of  $10.3 \pm 4.2 \mu\text{M}$  to  $2.0 \pm 1.3 \text{ nM}$  when  $10 \mu\text{M}$  carbachol is present (Albuquerque et al., 1979). Likewise, it has been reported that submicromolar concentrations of carbachol enhance the initial rate of  $[^3\text{H}]\text{-PCP}$  binding to electroplax membranes by up to two orders of magnitude (Aronstam et al., 1980). These researchers also find a five-fold decrease in the  $K_d$  for PCP inhibition of radiolabelled  $\text{H}_1\text{-HTX}$  (a ligand with selectivity for the nicotinic ion channel) binding in the presence of  $1 \mu\text{M}$  carbachol. Thus, our physiological findings are in agreement with biochemical evidence that agonist interaction with receptor causes an enhanced affinity of PCP for a channel binding site.

Adiphenine did not cause a decremental response with repetitive agonist challenge (figure 16) and gave similar results with both the sequential and the cumulative variations of the concentration of carbachol (figure 15). Although these findings do not give any evidence for a selectivity of adiphenine towards open or closed channels, these results do not eliminate this possibility. It must be noted that if the

equilibrium of the drug with channels of both varieties is rapidly obtained (within several minutes) the techniques used here would be incapable of demonstrating channel selectivity. In the case of PCP the dissociation rate from binding to open channels is sufficiently slow for the difference in affinity between the two states of channels to be observed.

Prolonged treatment with agonist alone results not only in an agonist activated-open channel conformation of the receptor channel complex but a desensitized-closed channel conformation as well. It therefore was of interest to examine whether the agonist enhancement of PCP inhibition may be due to enhancement of agonist induction of the desensitized conformation. If this were so, conditions which enhance desensitization to agonist alone should increase the rate and possibly the degree of PCP antagonism. Two factors which are established to enhance the rate of desensitization are increasing agonist (Rang and Ritter, 1970) and  $\text{Ca}^{++}$  (Manthey, 1966) concentration. Using the fixed schedule agonist addition technique desensitization is observed in control muscles to successive doses of 15 $\mu\text{M}$  carbachol (figure 20). This concentration does not enhance the rate or extent of PCP antagonism to any greater extent than a lower agonist concentration (7.5 $\mu\text{M}$ ) which is not desensitizing by itself. When desensitization is increased by raising the concentration of  $\text{Ca}^{++}$  (6.6mM), PCP antagonism is not significantly different from that observed with normal  $\text{Ca}^{++}$  (2.0mM, figure 21). These results support the suggestion that agonist induced increase in PCP blockade is not due to a desensitizing mechanism. Thus it suggests that PCP can distinguish different conformations of the nAChR-ion channel complex and that PCP exhibits its highest affinity for

the agonist activated-open channel conformation.

Although it would have been more desirable for data comparison to perform binding studies on the rectus abdominis muscle (the muscle employed for the physiological experiments) the relatively long duration of time required for binding experiments due to slow diffusion through the muscle made it impractical. Frog sartorius muscle is a much thinner sheet of muscle fibers than is the rectus abdominis. The muscle contains about 500 fibers; each having 2 to 4 endplates (Katz and Kuffler, 1941; Miledi, 1960). The first reports of [ $^{125}$ I]  $\alpha$ -BTX binding to intact sartorius muscle (Miledi and Potter, 1971) suggested that diffusional barriers did not prevent maximal toxin labelling of the muscle. Indeed, in the present experiments complete labelling of sartorius muscle was obtained with 10nM [ $^{125}$ I]  $\alpha$ -BTX in only 24 hours (figure 22) whereas maximal labelling of the rectus was quite variable and required from 3 to 7 days under identical conditions. Because saturation labelling of the sartorius is more consistent as compared to the rectus muscle, i.e., the total number of  $\alpha$ -BTX binding sites has little muscle to muscle variability ( $239.3 \pm 21.3$  fmoles/muscle, table 4), this preparation has proved to be a useful test system for binding studies.

Receptor sites were observed to be stable under the standard conditions selected for  $\alpha$ -BTX labelling of intact muscle (40 hrs at 4°C). Table 2 shows that muscles preincubated for 72 hrs at 4°C and freshly excised muscles have the same number of [ $^{125}$ I]  $\alpha$ -BTX sites when subsequently solubilized in Triton X-100 and toxin labelled ( $170 \pm 32.8$  and  $175 \pm 30.5$  fmoles/muscle, respectively). Toxin binding sites are also stable to degradation during the time course of detergent solubilization. The same number of labelled sites is obtained after 1 hour or 24

hours of detergent extraction (2% Triton X-100) of a homogenate from labelled intact sartorius muscle (figure 23). The  $\alpha$ -BTX binding is considered to be irreversible for all practical purposes because we could detect no appreciable dissociation of toxin bound to intact muscle within a 72 hour period. Extremely slow dissociation of bound  $\alpha$ -BTX is also suggested by the results of physiological studies. Miledi and Potter (1971) demonstrated that frog sartorius muscle incubated in  $10^{-5}$  g/ml ( $1.25\mu\text{M}$ ) for 2 hrs showed no recovery of neuromuscular transmission or ACh sensitivity after 8 days of washing even though muscle fibers remained directly excitable. The stability of receptors during toxin labelling, stability of the receptor-toxin complex over the duration of extraction, and a lack of any significant dissociation of toxin from the complex during assay establish the validity and reliability of binding studies utilizing intact sartorius muscle.

The distribution of  $\alpha$ -BTX binding sites in whole sartorius muscle indicates that 85-90% of the sites are probably on the muscle membrane and are accessible to labelling by bath applied  $\alpha$ -BTX (see table 3). A small but significant population (10-15% of total binding sites, i.e., 20-30 fmoles) is inaccessible to bath applied toxin and requires detergent solubilization to be labelled. The significance of this population of "hidden" receptors is unclear. Devreotes and Fambrough (1976) have similarly observed a "hidden" fraction of  $\alpha$ -BTX binding sites in myoblast tissue cultures which represent approximately 15% of the total sites. They have localized these sites by electron microscopy to internal membranes, including those of secondary lysosomes, and have presented evidence that they are receptors internalized from the surface

and not a precursor population of surface receptors.

Although diffusion of  $\alpha$ -BTX throughout the muscle is much faster in the sartorius as compared to the rectus abdominis, diffusion is probably rate limiting for  $\alpha$ -BTX binding to whole sartorius muscle nAChR (Miledi and Potter, 1971). Due to this problem, analysis of the kinetics of toxin binding to receptors in intact muscle does not directly characterize the nature of the binding reaction. For this reason toxin binding was measured after a fixed time interval and under standard conditions using varying concentrations of  $\alpha$ -BTX (See Materials and Methods, Section 2.E.). Results obtained in this manner were analyzed by the method of Scatchard analogous to the analysis of equilibrium binding data for fully reversible ligands. However, interpretation of the data obtained in this way is not the same as if a true equilibrium of ligand and binding site existed. It must be emphasized that the numerical values obtained for affinity constants (pseudoequilibrium constants) for  $\alpha$ -BTX binding determined in this manner are not true affinity constants, rather, they are proportional to the true kinetic rate constants. These values thus represent relative affinities which only have validity for comparison with results obtained from other experiments in which toxin binding is performed under identical conditions.

Binding of  $\alpha$ -BTX to intact muscle is best described by a single affinity kinetic class of sites (figs. 24-27). Comparison of results obtained for DPA alkylated muscle with that of control preparations (table 4) suggests that the toxin binding site and the agonist binding site are distinct from one another in the membrane-bound receptor but the former may include the latter. This conclusion is based on the

observation that DPA alkylation results in no loss in the number of  $\alpha$ -BTX sites on the muscle ( $240 \pm 16.0$  fmoles as compared with  $239.3 \pm 21.3$  and  $232 \pm 12.0$  fmoles in untreated and DTT reduced muscle, respectively), although it does affect the affinity (rate) of toxin for the receptor (pseudoequilibrium constants equal  $45.8 \pm 13.8$ ,  $3.45 \pm .17$  and  $2.50 \pm .26$  nMolar for DPA alkylated, DTT reduced and untreated muscle, respectively). One possible explanation is that DPA alkylation affects the  $\alpha$ -BTX binding site in an indirect way, possibly by inducing a conformational change in the toxin binding site via an allosteric mechanism. An alternative explanation for the reduced affinity of  $\alpha$ -BTX to DPA alkylated muscle is that  $\alpha$ -BTX binding normally involves multipoint attachment to the receptor and the agonist binding site represents only one of these sites. Permanent occlusion of the agonist site by DPA alkylation would eliminate only one of the several binding sites for toxin on the receptor and result in a reduction in affinity but not number of sites available. Although our data do not rule out the latter possibility, the former explanation (allosteric influence) is suggested by physiological findings that DPA alkylation of reduced muscle irreversibly locks the nAChR in an agonist activated conformation. It is further consistent with a large body of evidence which indicates that simple cleavage (via reduction) of a disulfide bond in the vicinity of the agonist binding site on the nAChR causes significant changes in potency as well as intrinsic activity of responses to nicotinic cholinergic drugs (Rang et al., 1971; Karlin, 1968; Karlin, 1969; Ben-Haim et al., 1973). The observation of simultaneous occupancy of the nAChR by both DPA and  $\alpha$ -BTX is consistent with a growing body of evidence that agonists and snake  $\alpha$ -toxins may compete with one another by an allo-

steric mechanism (Sarvey et al., 1978; Bulger and Hess, 1973; Fu et al., 1974; Hess et al., 1975) and not by direct competition at one site.

The site of DPA alkylation which is responsible for the sustained agonism in physiological experiments appears to be the same as the site of alkylation which results in reduced affinity (rate) for toxin binding because of 1) the mutual requirement for sulfhydryl reduction and 2) the observation that MBA, another alkylating aziridinium species (an isomer of DPA) which does not behave as a nicotinic agonist and has little affinity for the receptor (see Results, Section 14) does not reduce the affinity of toxin binding to membrane bound nAChR (table 4). More convincing evidence supporting the above conclusions that DPA alkylates the agonist site on the nAChR comes from binding studies with agonists and antagonists competing for toxin binding.

Curare was found to be equipotent as an inhibitor of  $\alpha$ -BTX binding in both untreated and DPA alkylated membrane bound nAChR; however, DPA alkylation significantly reduces the ability of carbachol to compete for toxin binding (figs. 28 and 29, summarized in table 5). This finding is interpreted to mean that when the receptor is occupied by DPA, the agonist binding site is occluded and the addition of carbachol can cause little further reduction in the affinity (rate) for  $\alpha$ -BTX binding. The fact that DPA alkylation of the agonist binding site does not similarly alter the potency of dTC, an antagonist, to compete for  $\alpha$ -BTX binding provides direct evidence for a physical distinction between binding sites for agonists (carbachol) and antagonists (dTC) on the nAChR of intact sartorius muscle. In order to explain the apparent competition for receptor binding sites by agonists and antagonists found here and reported by other investigators (Colquhoun and Rang, 1976; Brockes and

Hall, 1975; Barnard et al., 1977) in terms of a separate agonist and antagonist site, one must postulate that the binding of either species induces a conformational change in the receptor such that the binding site of the other species has reduced affinity for its ligand, i.e., the binding sites interact allosterically with one another. Alternatively the sites may partially overlap.

In spite of its potency as an antagonist of neuromuscular response, PCP has little ability to compete for the binding of  $\alpha$ -BTX to the membrane bound nAChR (see table 5). These findings are in agreement with results obtained by Kloog et al. (1979) demonstrating that PCP, at concentrations which blocked the response of frog rectus abdominis muscle to ACh, did not block  $\alpha$ -BTX binding. Electrophysiological studies indicate PCP to be a potent ligand for the nicotinic receptor ion conductance channel (Tsai et al., 1979; Albuquerque et al., 1980). Even though the channel may comprise one or more of the subunits of the nAChR complex, we were unable to detect an allosteric effect of PCP on the membrane bound acetylcholine receptor by changes in  $\alpha$ -BTX binding parameters.

One significant finding in the present investigation is the difference in characteristics for toxin binding to intact and soluble nAChR. Unlike toxin binding to intact muscle nAChR, a single class of binding site cannot explain the concentration dependence of  $\alpha$ -BTX binding during a fixed interval (24 hrs) to solubilized receptors from untreated, reduced; or DPA alkylated sartorius muscle (figs. 30, 31, and 32). The non-linear Scatchard plots obtained from pseudoequilibrium experiments prompted a detailed kinetic analysis of  $\alpha$ -BTX binding to solubilized receptors. Statistical methods were used to establish that two

independent binding sites is the simplest model which will account for the data (table 10 and 11). The fast toxin association rate constant was calculated to be 20-fold larger than that of the slow binding site. Other groups have also observed two kinetic sites for toxin binding to solubilized nAChR from innervated and denervated vertebrate muscle (Mittag et al., 1978; Kemp et al., 1980; Brockes and Hall, 1975; Almon and Appel, 1976). By contrast to the 20-fold difference in the two association rate constants observed here for  $\alpha$ -BTX binding to frog muscle nAChR, Mittag et al. (1978) and Kemp et al. (1980) observed a 7-fold difference while Brockes and Hall find a 4-fold difference in rate constants for the two  $\alpha$ -BTX binding sites of rat muscle nAChR. An extreme difference in kinetic rate constants of 4 orders of magnitude, as reported by Almon and Appel (1976) in solubilized denervated muscle, suggests the possibility of an additional extremely low affinity set of toxin binding sites which would not have been observed in the concentration range of toxin employed in this and other studies cited above. In agreement with Mittag et al. (1978) and Brockes and Hall (1975) we find the two kinetic sites to be present in approximately equal amounts in untreated frog muscle.

The basis for the subdivision of an apparently homogeneous population of  $\alpha$ -BTX binding sites in an intact muscle into two kinetically distinguishable populations following detergent extraction from the membrane is intriguing. Removal of some membrane induced constraint may allow an asymmetry to develop between  $\alpha$ -BTX binding sites on nAChR. It is an open question whether these two sites for toxin binding in muscle extracts reside on a single nAChR molecule or reside on two distinct populations of nAChR molecules. The 1:1 ratio of fast to slow toxin

binding sites observed here and reported by others (Mittag et al., 1978; Brockes and Hall, 1976) could indicate two non-identical sites on a single nAChR molecule. However, the physical separation of two distinct toxin binding sites with the aid of lectin affinity chromatography and by anti-nAChR antibody from myasthenic sera suggests that different receptor molecules (Mittag et al., 1978) contain kinetically distinct toxin binding sites.

Dissociation of toxin from solubilized nAChR was observed to be extremely slow with only a maximum of 13% occurring after 92 hours. Assuming a simple first order process, an upper limit for the dissociation rate constant was calculated to be  $2.65 \pm 3 \times 10^{-5} \text{ Min}^{-1}$  (figure 33). The estimated value takes into account not only dissociation but also any loss due to receptor degradation as well. The extremely slow dissociation of the  $\alpha$ -BTX-nAChR complex observed here is consistent with the results of Colquhoun and Rang (1976) who found no measurable dissociation within 45 hours and those of Kemp et al. (1980) who observed a  $T_{1/2}$  for  $\alpha$ -BTX dissociation of 70-100 hours for receptors extracted from innervated and denervated muscles. In contrast biphasic dissociation with a  $T_{1/2}$  of 3.5 to 4.0 hours and 100 hours for each of two approximately equal populations was reported by Brockes and Hall (1975). Utilizing the rate constant for dissociation and the measured association rate constants for toxin binding from table 10, the maximum values for the equilibrium constants for  $\alpha$ -BTX binding to solubilized frog sartorius nAChR are  $5.2 \times 10^{-12} \text{ M}$  and  $1 \times 10^{-10} \text{ M}$ , respectively, for the two components.

$\alpha$ -BTX binding experiments done at short time intervals or employing relatively low concentrations of  $\alpha$ -BTX would give preferential labelling

of the fast associating binding component. The results obtained by both of these methods indicated that the affinity of the fast toxin binding component is the same for solubilized receptors prepared from DTT reduced, DPA alkylated, and from untreated control sartorius muscle preparations (tables 6 and 10). In addition, analysis of the slow kinetic rate constant for toxin binding was found to be unaffected by reduction or DPA alkylation (table 10). However, the significant effect of these muscle treatments on the parameters of toxin binding to soluble receptor is a selective loss in the number of fast associating  $\alpha$ -BTX binding sites following DTT treatment ( $41.0 \pm 10.1\%$ ) and DPA alkylation ( $53.5 \pm 6.1\%$ ). These findings for soluble nAChR are in direct contrast to the binding results obtained with membrane bound receptors in which the number of toxin binding sites was unchanged and a large decrease in the affinity of  $\alpha$ -BTX was observed ( $>10$ -fold) subsequent to DPA alkylation (but not with DTT reduction alone). This suggests that the membrane bound nAChR after DPA alkylation is under some conformational constraint (the agonist activated conformation) characterized by a reduced rate of toxin binding. This constraint is removed by solubilization with Triton X-100. Extraction of the agonist-activated conformation of nAChR (DPA alkylated) causes the loss of this conformational state and/or conversion to other states which have rate constants for  $\alpha$ -BTX binding indistinguishable from those observed in receptor extracted from untreated muscles (resting conformation of nAChR). However the state(s) assumed by some of the DPA alkylated nAChR when solubilized preclude toxin binding which results in a loss of fast toxin binding sites. Conditions of DPA alkylation which decreased the toxin binding affinity of almost all of the membrane bound receptors cause a loss of only 25%

of total receptors when solubilized (50% of the fast associating and none of the slow associating form).

If the two forms of toxin binding sites occur as independent sites on the same divalent nAChR molecule in the membrane bound state, then DPA may be expected to affect a maximum of either none, half or all toxin binding sites when the effect of DPA is subsequently determined by analyses of solubilized receptors. The observation that at a maximum 25% of all sites are affected (half the fast sites) only suggests the existence of more than one protein form of the nAChR and that DPA affects only receptor molecules bearing fast sites when determined on solubilized receptors.

Whatever interpretation is favored, these results clearly show that toxin binding sites that appear to be equivalent under some conditions can be converted to distinguishable subpopulations, i.e., in other experiments at least two classes of  $\alpha$ -BTX sites are present.

Carbachol and dTC compete effectively for both the fast and the slow toxin binding sites in solubilized sartorius nAChR. This is shown graphically in figure 34, which utilizes conditions of [BTX], [AChR], and time such that essentially all of the fast toxin binding sites and greater than 50% of the slow sites are labelled with [ $^{125}$ I]  $\alpha$ -BTX in the absence of competitor (calculated from kinetic data in table 10).

Although both kinetic populations of  $\alpha$ -BTX binding sites were inhibited by carbachol and dTC, the Hill coefficients for the competition curves, 0.68 and 0.66, respectively, suggest the possibility of a differential affinity for both competitors at the two sites. Under these conditions carbachol and dTC had apparent  $IC_{50}$  values for inhibition of toxin binding in the micromolar range, the range in which they exert their

biological effects on frog neuromuscular preparations (see table 8). PCP, on the other hand, had little effect on toxin binding with an  $IC_{50}$  of approximately 1 millimolar, 2-3 orders of magnitude greater than the  $IC_{50}$  for the inhibition of carbachol induced contraction of the rectus abdominis muscle. The shallow slope of the PCP inhibition curve for toxin binding ( $<.5$ ) relative to those found for carb and dTC (.676 and .657, respectively) is consistent with an indirect, possibly an allosteric, mode of inhibition of toxin binding.

Following DPA alkylation of the muscle the ability of dTC and carbachol to inhibit toxin binding to solubilized nAChR is significantly reduced (15-fold and 4-fold relative to untreated nAChR preparations; see table 8). A loss in potency for antagonism of toxin binding was observed in DTT reduced muscle as well, but to a lesser extent (4-fold and 2-fold for carbachol and dTC, respectively). In contrast, PCP was equieffective as an inhibitor of  $\alpha$ -BTX binding in untreated, DTT reduced and DPA alkylated preparations. One possible explanation is that reduction and DPA alkylation modify the conformation of the receptor by an action at the agonist and antagonist binding sites but not at the PCP binding site. It is also possible that the specific binding sites for agonists and antagonists are not directly affected by these treatments but that a conformational transition in the receptor resulting from the binding of carbachol and dTC which is necessary for a decrease in the rate of toxin binding is prevented by reduction and alkylation. A third possibility is that DPA alkylation and to a lesser extent DTT reduction selectively eliminate the fast toxin binding site from the nAChR population as determined on solubilized receptor (see table 10). These experimental results may arise if both dTC and carbachol have a higher

affinity for the fast toxin binding sites which have been progressively eliminated. Elimination of the higher affinity site for these ligands by reduction or DPA alkylation would require a greater concentration of the ligands to occupy their lower affinity sites in order to achieve 50% inhibition of total sites; i.e., an apparent increase in  $EC_{50}$  would be expected. Although the former possibilities cannot be completely ruled out (i.e., they may contribute partially to the effect), the possibility that agonists and antagonists have a higher affinity for the fast toxin binding component is strongly indicated by these results.

The influence of carbachol, dTC, and PCP on toxin binding during a short time interval of toxin binding (3 hrs as opposed to 24 hrs) allowed selective study of the ability of these ligands to compete selectively for the fast toxin associating species of receptor (fig. 38, table 9). Under the conditions employed (in the absence of competing ligand), the fast associating sites account for more than 80% of the total binding determined. A comparison of the results obtained at 3 hrs with those for 24 hrs of toxin incubation indicates that the change in  $EC_{50}$  for carbachol and dTC resulting from DPA alkylation is small or absent, when looking at the fast toxin sites alone. Although DPA treatment alone selectively eliminates a fraction of  $\alpha$ -BTX binding sites measurable in a 3 hr competition experiment (i.e., it reduces the 100% toxin bound value), the experimental conditions are such that the fast sites which remain can still be selectively labelled and identified by their rate constant. Thus, there is little or no effect of DPA alkylation on the ability of carbachol and dTC to compete for toxin binding to these fast sites. When similar experiments are performed after a 24 hr toxin incubation, the DPA dependent loss of fast toxin

binding sites results in an increased proportion of slow binding sites in the remaining receptor population. It is under these conditions (i.e., those favoring slow toxin binding sites) that DPA dramatically affects the apparent  $IC_{50}$  values for carbachol and dTC inhibition of toxin binding. This finding suggests that either the affinity of carbachol and dTC is intrinsically less for the slow toxin binding site than the fast one or that DPA treatment specifically and selectively reduces the affinity of agonists and antagonists for the slow toxin binding sites in addition to eliminating the fast sites. Results of kinetic studies confirm the former hypothesis and rule out the latter.

Contrary to reports of kinetic studies which suggest competitive inhibition by carbachol and dTC for  $\alpha$ -BTX binding to detergent extracted nAChR (Colquhoun and Rang, 1976; Brookes and Hall, 1975) our studies suggest that neither of the kinetic rate constants for toxin binding is affected by these ligands but that their effect is to eliminate some population of the sites from the binding reaction. At  $10\mu M$  concentrations of carbachol and dTC there is no significant effect on the kinetics of toxin association, but both agents cause a selective decrease in the number of fast toxin associating sites (tables 12 and 14). Thus, these ligands exhibit selectivity at low concentrations for the fast binding sites. The fractional disappearance of this component depended on the concentration of the competing ligand and occurred in preparations derived from control, reduced and DPA alkylated muscle (tables 12-15). More than 90% of the fast toxin binding sites were lost in the presence of  $75\mu M$  carbachol or  $25\mu M$  dTC but there is no effect at these ligand concentrations on the rate of the slow toxin binding component, whereas at much higher concentrations, carbachol and dTC

clearly will inhibit all toxin binding sites, namely 1mM concentrations inhibit >90% of total toxin binding (figure 34).

Although other laboratories have reported that carbachol (Colquhoun and Rang, 1976) and dTC (Colquhoun and Rang, 1976; Brookes and Hall, 1975) competitively inhibit  $\alpha$ -BTX binding in solubilized muscle nAChR, their kinetic analysis did not cover the full time course. Instead, the conclusions of both groups are based on measurement of the depression of the initial rate of toxin binding which gives no indication of what the final concentration of toxin receptor complex would have been in the presence of competing ligand. The apparent depression in the initial rate of toxin binding, assumed to be due to competition, would also have been expected if binding sites were actually eliminated. Furthermore,  $\alpha$ -BTX binding under the experimental conditions that were used in these studies would have labelled only a fraction of the fast toxin binding sites and an insignificant number of the slow ones. Neither of these groups evaluated the time course of toxin binding in the presence of competing ligand over the protracted period of time necessary to directly measure the  $B_{max}$  value for toxin binding. Had this been done a decrease in  $B_{max}$  for toxin binding would have been observed. Thus, the more detailed findings reported here, although subject to a different interpretation, are not inconsistent with the actual results obtained in these other laboratories.

The hypothesis presented here that carbachol and dTC, both reversibly acting cholinergic ligands, can cause the apparent elimination of sites for the irreversibly binding  $\alpha$ -BTX because they are behaving as irreversible ligands is an intriguing one. In order to explain this it may be postulated that either of these competitors when

bound to the receptor irreversibly block the association of toxin or greatly enhance the rate of its dissociation from the receptor complex. The first possibility is ruled out since clearly carbachol and dTC do not bind irreversibly to the nAChR by covalent bond attachment. As reversible receptor ligands the finite dissociation rate of these bound ligands from receptor should result in the eventual labelling of all sites by [ $^{125}$ I]  $\alpha$ -BTX given sufficient time. However, within the time frame of the kinetic experiments reported here, approximately 5000 minutes, we find no gradual time dependent appearance of these missing sites. They appear to have been simply eliminated from the binding reaction.

Kinetic experiments show that PCP, in common with carbachol and dTC, resulted in the selective loss of the fast component of toxin binding to solubilized nAChR at 1  $\mu$ M PCP (table 17). However, the high concentrations of PCP relative to carbachol and dTC necessary to achieve this effect suggested that it may be the result of PCP binding to the receptor recognition site rather than to the channel component.

PCP was observed to increase the  $IC_{50}$  value measured for carbachol and dTC inhibition of  $\alpha$ -BTX binding after a fixed period of time (table 7). Since carbachol and dTC are much more potent inhibitors of the fast as opposed to the slow toxin binding sites, the selective inhibition by PCP of the fast toxin site and consequent enrichment of the receptor population in slow sites can explain this finding. As the proportion of slow toxin binding sites increases in the reaction mixture (resulting from selective PCP inhibition of fast sites) increasing concentrations of carbachol or dTC should be required to inhibit 50% of toxin binding. This relationship between inhibition of sites by PCP and

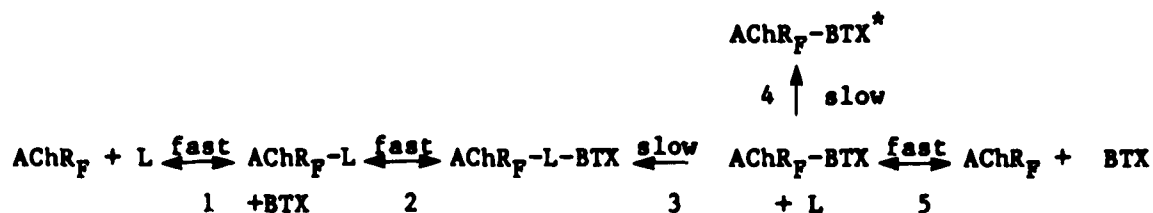
increasing EC<sub>50</sub> of the remaining sites for carbachol inhibition of toxin binding which we have observed (figure 37) is consistent with this notion. Because of the above, it is not necessary to invoke a hypothesis of an indirect effect (possibly allosteric) on the agonist and antagonist binding sites caused by PCP binding to the channel in soluble nAChR.

The observed loss of fast toxin binding sites induced by carbachol occurred when toxin-receptor complex was assayed by the gel filtration assay as well as by the Con A assay. Since the gel filtration method does not involve any adsorption or other molecular interaction between the gel and the receptor complex, this finding rules out the possibility that the interaction of receptor with the Con A during the assay itself was involved in the loss of toxin from some binding sites. There was some possibility that the Con A could have affected the results since previous reports noted toxin binding inhibition by prior treatment of receptors with soluble Con A of all (Almon and Appel, 1976) or half of the  $\alpha$ -BTX binding sites in muscle extracts (Mittag et al., 1978). The gel filtration result shows that the observed loss of fast binding sites for  $\alpha$ -BTX is not an artifact generated by the specific method employed for separation of toxin-receptor complex from the free toxin (the Con A assay).

If a ligand enhanced rate of dissociation is responsible for the apparent loss of fast toxin sites, it must occur within the time span of approximately 30 minutes at 4°C, i.e., the period of time from removal of the test sample from the incubation to the final column wash which is required for the Con A assay of receptor-toxin complex (Materials and Methods, Section 2.B.). Implicit in this hypothesis is the formation of

ternary complexes involving the nAChR,  $\alpha$ -BTX, and the competing ligand in which dissociation of the latter is slow or absent while dissociation of the former is greatly increased. The existence of such ternary complexes has previously been suggested by a number of groups to explain observations with the electroplax nAChR (Bulger and Hess, 1973; Eldefrawi and Eldefrawi, 1973; Fu et al., 1974; Hess et al., 1975). Carbachol and dTC may enhance dissociation of preformed receptor-toxin complex or, alternatively, it might be necessary that they bind to receptor prior to  $\alpha$ -BTX for enhanced dissociation of toxin to occur, i.e., the order of ligand binding to the receptor may be important as it is for Con A inhibition of  $\alpha$ -BTX binding. The results of preliminary experiments show that neither carbachol, dTC, nor PCP enhance the rate of dissociation from preformed receptor-toxin complex, indicating that the competing ligand must bind to the receptor prior to or simultaneously in order to achieve this effect. The most reasonable explanation of our findings is that the receptor subpopulation which is bound by agonist, antagonist, or in the presence of high concentrations of a channel blocker (PCP) can bind  $\alpha$ -BTX reversibly at the fast binding site but do not form an irreversible complex with toxin. This requires postulating a two-step model for the normal mechanism of toxin binding whereby a rapid reversible step precedes the formation of an irreversible complex. Such two-step models for  $\alpha$ -BTX binding have been proposed previously to explain toxin binding kinetics to purified nAChR (Klett et al., 1973; Bulger and Hess, 1973).

The following reaction scheme is proposed to account for the apparent loss of fast toxin binding sites in the presence of carbachol, dTC, and PCP (designated by L) in solubilized receptor preparations:



AChR<sub>F</sub> is the fast toxin binding component of AChR, and an (\*) denotes the formation of an irreversible complex.

The binding reactions designated as fast are considered to be relatively rapid and reversible. Conversion of a reversibly bound receptor-toxin complex to an irreversibly bound form is considered to be slow as is the association of L to form the ternary complex. When the nAChR is bound with L, α-BTX can be bound only reversibly to the receptor, i.e., the slowly developing irreversible association (step 4) cannot occur. Additionally, when α-BTX binds to the L occupied receptor, L can no longer dissociate perhaps because of steric impedance by the large size of the toxin molecule (step 3), whereas normally L dissociates rapidly from the nAChR. α-BTX dissociation does occur from the L occupied ternary complex, the equilibrium conditions favoring the ternary complex formation in the binding reaction. The net result of this scheme is that essentially only the fast toxin binding sites which were free of L at the moment α-BTX was added to the incubation medium (time zero) can proceed to form an irreversible complex which is what is measured by the Con A or Sephadex assay at some later time. The reversible complexes existing at equilibrium dissociate the toxin during the separation of bound from free and thus do not appear as labelled receptor in the assay.

The physiological studies of Sarvey et al. (1978) are consistent

with this reaction scheme for the fast toxin binding site. Sarvey demonstrated that  $\alpha$ -BTX can cause an essentially complete inhibition of muscle responsiveness in the presence of agonists such as carbachol and decamethonium. However, the nature of the toxin inhibition is changed by the presence of agonist. This is shown by the observation that even though these agonists (at the concentrations employed) could not protect the muscle from a direct inhibitory action of  $\alpha$ -BTX, they did prevent the irreversible action of  $\alpha$ -BTX which would have occurred in their absence. Thus under these conditions, the toxin inhibition was reversed by washout. These experiments clearly demonstrate simultaneous occupancy of the receptor by  $\alpha$ -BTX and a competing ligand. Furthermore, they show that a reversible and an irreversible two-step mechanism is likely for toxin binding to the nAChR. In the presence of agonist toxin may bind to the occupied receptor and block the receptor mediated response, but the irreversible secondary binding reaction of toxin to the receptor appears to be precluded. These findings also suggest that the toxin binding characteristics of the nAChR in vivo are more similar to the fast toxin binding sites than the slow ones in solubilized receptor preparations.

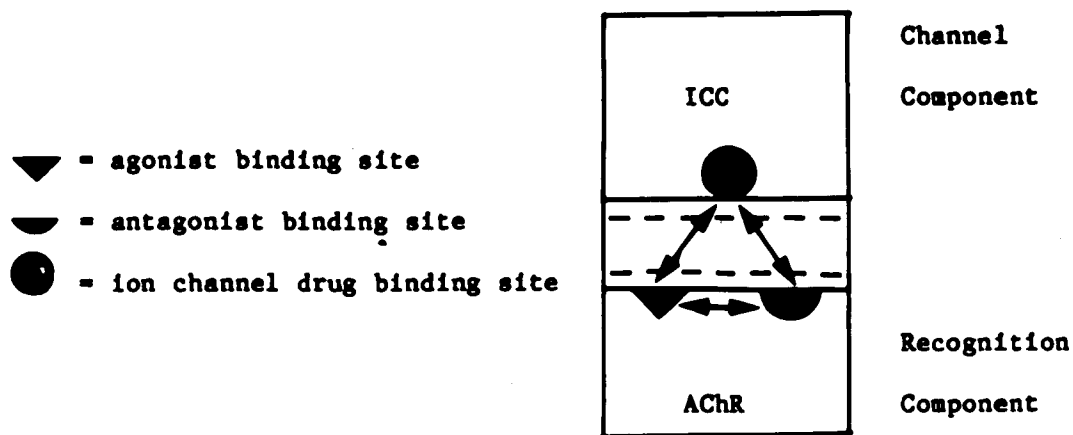
The significance of the slow toxin binding sites found in soluble nAChR preparations is presently unclear. It is not known whether this population of sites reside on the same nAChR molecule as do the fast binding sites or if they are present on distinct nAChR molecules. Although carbachol and dTC do inhibit toxin binding to the slow sites they do so only at high concentrations (figure 34). The nature of this inhibition is not known since kinetic studies were not performed on  $\alpha$ -BTX binding at these high concentrations of carbachol and dTC. Thus, it is not known whether these ligands will affect the kinetic rate constant or

the total number of slow sites as was the case for fast sites. This is an area worthy of future investigation.

From the outset of this study, we were aware that the most precise measure of possible allosteric actions of ligands on the nAChR would be from analysis of the altered kinetics of  $\alpha$ -BTX binding. Since there is a diffusional limitation of toxin labelling of intact muscle receptor which precludes direct kinetic experiments it was hoped that kinetic studies on the soluble receptor could be extrapolated to the membrane bound (functional) situation and explain the findings on whole muscle. The experimental results clearly indicate that one cannot generalize findings on soluble receptor to the membrane bound nAChR and that extraction of the receptor from its membrane environment causes major changes in the binding properties for toxin and small ligands. The magnitude of these changes indicates that, for all practical purposes, receptor in the two forms (detergent solubilized or membrane bound) should be regarded as distinct entities with regard to ligand binding reactions. Removal of the receptor from its membrane environment results in 1) the subdivision of an apparent single class of  $\alpha$ -BTX binding sites into two classes of sites; 2) qualitative changes in the effect of DPA on the parameters of toxin binding; 3) quantitative changes in the ability of carbachol and dTC to differentially interfere with toxin binding in control and DPA alkylated muscle. While it was not necessary to invoke a hypothesis of allosteric interaction between binding sites on the receptor complex for agonists, antagonists and ion channel blocking ligands to explain results obtained with soluble receptor preparations, this was necessary to explain the findings on the membrane bound system. The disparity between findings with soluble and membrane bound nAChR point to the general

problem of extrapolating results of binding studies utilizing a solubilized form of a conformationally labile membrane protein to infer properties of the same protein as part of an intact system.

A number of features of the allosteric model for the functional nAChR complex (previously presented in Introduction, Section 5) have been verified by our experimental findings with intact muscle. The model suggests that there are separate binding sites on the receptor-complex for each of the three functional classes of ligand (agonist, antagonist, channel blocker). According to the proposed model, the binding of ligand to any one of the three sites may influence the other two sites via conformational transitions of receptor subunits which are linked to one another as shown diagrammatically.



Direct evidence for the existence of distinct sites for agonists and antagonists is provided by the finding of a decreased potency for inhibition of toxin binding by carbachol but not by dTC, in DPA alkylated muscle (relative to control muscle). Since occlusion of the agonist site

by alkylation of the receptor does not diminish the ability of dTC to bind to the receptor and thereby to interfere with toxin binding in contrast to the inhibitor actions of carbachol, these ligands must bind at distinct or partially distinct sites on the receptor. If the receptor binding sites for carbachol and dTC are distinct, the well known competitive interaction between these ligands observed in physiological experiments must now be explained by an allosteric mechanism. Evidence that ligand binding to the antagonist site can allosterically influence the agonist binding site is provided by the observation that dTC protects against DPA alkylation. However, a similar mechanism might result from a channel blocking effect of dTC at the high concentration of dTC used in these experiments.

Drugs which appear to be selective for the ion channel (PCP and adiphenine) gave experimental results indicating that they allosterically influenced the conformation of the agonist site on the recognition component of the receptor. Their ability to protect against contracture resulting from DPA suggests that they either inhibit the binding of DPA to the agonist site or they alter the proximity of the alkylatable SH group to the agonist site. Conversely, when agonist is bound to the recognition component of the receptor it seems to result in a conformational change in the channel such that PCP has a higher affinity for binding to the channel drug binding sites than in the absence of agonist. Experimental evidence for this is the enhanced inhibition of agonist induced muscle tension by PCP in the continuous presence of agonist.

Although results on ligand competition with toxin binding in soluble receptors could have been explained without invoking an allosteric

mechanism for ligand effects on toxin kinetics, this does not necessarily imply that such mechanisms are ruled out. These studies could be extended to detailed competition experiments using various combinations of agonists, antagonists, and channel blockers. The results of such studies might yield more detailed information on this point. Another area for future investigation would be a study of the ligand competition for the slow toxin binding sites. Some insight might be gained as to a possible biologic relevance for this class of toxin binding sites.

However, these projects are extensions from the present investigations and are the subjects of future studies.

## SUMMARY AND SIGNIFICANCE

The nAChR complex at neuromuscular junction serves two functions, that of signal recognition in response to ACh and that of mediating the permeability response to that signal. In a functional sense the receptor can be considered to be composed of two components, a recognition protein which has binding sites for agonists and antagonists (i.e., ACh, dTC, snake venom toxins) and an ion conductance channel component linked to the recognition site. The ion conductance channel binds a distinct group of drugs (i.e., local anesthetics, histrionicotoxin and phencyclidine) which alter the time course and amplitude of the passage of ions through the channel and thereby affect the characteristics of the nAChR system (endplate current). These drugs do not bind directly to the recognition component in the concentration range of their biological activity but probably behave as allosteric effectors to the recognition component.

The two categories of ligand that bind to the recognition component (agonists and antagonists) have been generally considered as competitors for a single binding site on the recognition protein. However, a mutual interaction between two distinct (or partially overlapping) binding sites for each of these two classes of ligand that behave as allosteric effectors of each other may not be ruled out. Thus there is the possibility of at least three distinct drug binding sites on the receptor complex corresponding to the three functional classes of ligand with which the receptor is known to interact.

Based on the models of allosteric enzymes, the binding of a ligand to a site on one protein subunit of a macromolecular complex such as the nAChR may induce an alteration in the conformation of other subunits and

thus change the ligand binding properties at some distant site. Evidence obtained from binding studies using electropex membranes strongly indicated that the nAChR complex undergoes such allosteric transitions. The same allosteric relationships may be present in skeletal muscle systems and may be detected by ligand probes which are sensitive to the conformational state of the protein subunit which contains the binding site.

This study of the nAChR complex from frog skeletal muscle was an attempt to use ligand binding properties to detect conformational changes induced by the binding of a prototypic agonist (carbachol), an antagonist (d-tubocurarine), and an ion channel blocker (phencyclidine). The ligands and their effects were first characterized at the level of the physiological response (muscle tension) as well as the molecular level by binding studies. In addition to reversible ligands, this study employed DPA, an alkylating agent which we have found to be an irreversible agonist in reduced frog rectus abdominis muscle. The advantage of an irreversible agent is that it enables one to study the nAChR complex in a ligand induced conformation that has been fixed by the irreversible drug. Although it would also have been highly advantageous to have an irreversibly acting antagonist and an irreversible ion channel blocking component, these were not available. [ $^{125}$ I]  $\alpha$ -BTX was employed as the detector of indirect conformational changes resulting from the binding of small ligands of each of the three classes. The extremely high specificity of  $\alpha$ -BTX binding for the receptor as well as its relatively large size, making multipoint attachment likely, suggest that it would be sensitive to very subtle changes in conformation of the receptor subunits which would affect its binding parameters. Toxin

binding to the nAChR in the absence and presence of the three classes of ligand was studied on receptors in whole intact muscle as well as in detergent extracted nAChR for comparison.

DPA is a reversible cholinergic agonist on the frog rectus abdominis preparation consistent with its structural analogy to phenyltrimethyl ammonium. Following reduction of the muscle with DTT, however, DPA has an irreversible component to its response presumably due to alkylation of a reducible disulfide bond which has been well established to be in the vicinity of the acetylcholine binding site on the receptor. The agonist characteristic of the drug was blocked by pretreatment with BTX ( $2 \times 10^{-7}$  M) or with dTC ( $10^{-6}$  M). Furthermore carbachol ( $10^{-5}$  M) and dTC protected the muscle preparations from the irreversible response. Finally, the sustained DPA induced contracture is reverted by addition of the ion channel blocking ligands PCP (25  $\mu$ M) and adifenine (25  $\mu$ M). These findings are all in agreement that the sustained tension response resulting from DPA treatment of reduced muscle results from specific alkylation of the agonist binding site on the nAChR complex. Thus DPA serves as a means for converting the receptor into a stable "agonist" conformation so that the interactions with other ligands can be studied.

$\alpha$ -BTX and DPA can both occupy the nAChR complex simultaneously, i.e. a ternary complex can be formed with no loss in the number of toxin binding sites, but with a 20-fold reduction in the apparent affinity of the toxin. This suggests a significant change in the conformational state of the receptor protein as a result of DPA alkylation. Both  $\alpha$ -BTX ( $2 \times 10^{-7}$  M) and PCP revert the agonist response in DPA alkylated muscle. This finding provides further evidence that different ligands

can bind simultaneously for a mutual conformational interaction between the binding sites for  $\alpha$ -BTX and DPA on the nAChR complex.

Perhaps the most unexpected conclusion to come from these studies is that the agonist (carbachol) and antagonist (dTC) binding sites are distinct from one another on the receptor. Some direct evidence for this is provided by the finding that following DPA alkylation carbachol loses ability to compete for  $\alpha$ -BTX binding, whereas, the potency of dTC as a competitor is unchanged. This differential effect suggests that DPA alkylates a specific agonist binding site on the receptor but not the antagonist binding site. Addition of carbachol can do nothing to further interfere with  $\alpha$ -BTX binding because the site is already maximally occupied whereas this is not the case for the antagonist binding site. Analogous evidence for conformational interactions between the agonist binding site and the ion channel resultant from the binding of small ligands has been obtained. The channel ligand PCP (25 $\mu$ M) has no effect on the binding of  $\alpha$ -BTX, but protection is observed against DPA alkylation. This effect may be the result of a conformation change in the agonist binding site induced by PCP binding to its binding site on the channel. The protective effect could either be that DPA can no longer bind to the site or the proximity of the SH group has been altered so that DPA can bind but does not alkylate. In a reciprocal way, evidence was obtained which indicates that the binding of carbachol at the agonist site may influence the conformation of the channel component. Physiological studies indicate that PCP is a more potent inhibitor of the response to carbachol in the sustained presence of carbachol, i.e., it has higher affinity for the agonist activated conformation of the nAChR complex. Analysis of the data indicates PCP to

have a 2-3 fold higher affinity for the active than for the resting state.

Since the bulk of knowledge with regard to binding studies in skeletal muscle nAChR has been derived from solubilized muscle preparations, where applicable we replicated experiments performed on the intact muscle as described above with the detergent extracted preparation. Detergent extracted preparations also permit the more detailed kinetic analysis of toxin binding which is not possible with intact muscle due to diffusion limitations. Extraction of the nAChR complex from its membrane environment resulted in dramatic changes in ligand binding properties and/or the ability of ligands to induce conformational changes. Although  $\alpha$ -BTX binding data in intact muscle could be fitted to a single affinity class of binding sites, following detergent extraction the same total number of binding sites are recovered but they are best described by two kinetically distinct classes of site (20-fold difference in kinetic rate constants) existing in approximately equal number. Another major difference is that DPA alkylation reduces the affinity but not the number of  $\alpha$ -BTX sites for receptors in the membrane, but for solubilized nAChR DPA alkylation reduces the number but not the affinity of the sites. Furthermore the reduction in the number of sites is specific to the population of fast toxin binding sites and the slow sites are totally unaffected also in contrast to the membrane-bound receptors. Subsequent to DPA alkylation carbachol and d-tubo are both effective inhibitors of  $\alpha$ -BTX binding. These ligands are observed to have a substantially higher affinity for the first kinetic component of toxin binding in solubilized preparations. Whereas it was necessary to postulate allosteric interactions between ligand binding sites to

explain the data obtained for intact muscle, results from extracted muscle preparations could be explained by direct binding effects, although such allosteric mechanisms cannot be ruled out. These findings point out the dangers of extrapolating results obtained for the solubilized form of a membrane bound molecule, such as a receptor, to the membrane intact situation. A model is presented to account for the apparent irreversible inhibition of toxin binding (to the fast kinetic site) by carbachol and dTC which assumes the formation of a ternary complex and a two step toxin binding reaction scheme (an initial reversible phase followed by a slower essentially irreversible phase).

## BIBLIOGRAPHY

- Adams, P.R. Voltage jump analysis of procaine action at the frog end plate. *J. Physiol. Lond.* 268:291 (1977).
- Adams, P.R. A model for the procaine end-plate current. *J. Physiol. Lond.* 240: 61P-63P (1975).
- Adler, M., Oliveira, A.C., Eldefrawi, M.E., Eldefrawi, A.T., and Albuquerque, E.X. Tetraethyl ammonium: voltage-dependent action on endplate conductance and inhibition of ligand binding to post synaptic membranes. *Proc. Nat. Acad. Sci. USA* 76:531-535 (1979).
- Adler, M., Albuquerque, E.X., and Lebeda, F.J. Kinetic analysis of end plate currents altered by atropine and scopolamine. *Mol. Pharmacol.* 14:514-529 (1978).
- Albuquerque, E.X., Barnard, E.A., Porter, C.W., and Warnick, J.E. The density of acetylcholine receptors and their sensitivity in the postsynaptic membrane of muscle end plates. *Proc. Nat. Acad. Sci. USA* 71:2818-2822 (1974).
- Albuquerque, E.X., Barnard, E.A., Chiu, T.H., Lapa, A.J., Dolly, J.O., Jansson, S.E., Daly, J. and Witkop, B. Acetylcholine receptor and ion conductance modulator sites at the murine neuromuscular junction: Evidence from specific toxin reactions. *Proc. Nat. Acad. Sci. USA* 70:940-953 (1973).
- Albuquerque, E.X., Barnard, E.A., Jansson, J.-E., and Wieckowski, J. Occupancy of the cholinergic receptors in relation to changes in the endplate potential. *Life Sci.* 12:545-552 (1973).
- Albuquerque, E.X., Kuba, K., and Daly, J. Effect of histrionicotoxin on the ion conductance modulator of the cholinergic receptor: A quantitative analysis of the endplate current. *J. Pharmacol. Exp. Ther.* 189:513-524 (1974).

- Albuquerque, E.X., Tsai, M.-C., Aronstam, R.S., Eldefrawi, A.T., and Eldefrawi, M.E. Sites of action of phencyclidine II. Interaction with the ion channel of the nicotinic receptor. *Mol. Pharmacol.* 18:167-178 (1980).
- Albuquerque, E.X., Sokoll, M.D., Sonesson, B. and Thesleff, S. Studies on the nature of the cholinergic receptor. *Eur. J. Pharmacol.* 4:40-46 (1968).
- Almon, R.R., Andrew, C.G., and Appel, S.H. Acetylcholine receptor in normal and denervated slow and fast muscle. *Biochemistry* 13:5522-5528 (1974).
- Almon, R.R. and Appel, S.H. Cholinergic sites in skeletal muscle: interaction with Concanavalin A. *J. Neurochem.* 27:1513-1519 (1976).
- Almon, R.R. and Appel, S.H. Cholinergic sites in skeletal muscle II. Interaction of an agonist and two antagonists with the acetylcholine site. *Biochemistry* 15:3662-3667 (1976).
- Anderson, C.R. and Stevens, C.F. Voltage clamp analysis of acetylcholine produced endplate fluctuations at frog neuromuscular junction. *J. Physiol. Lond.* 235:655-691 (1973).
- Aronstam, R.S., Eldefrawi, A.T., Pessah, I.N., Daly, J.W., Albuquerque, E.X. and Eldefrawi, M.E. Regulation of [<sup>3</sup>H] perhydrohistrionicotoxin binding to torpedo ocellata electroplax by effectors of the acetylcholine receptor. *J. Biol. Chem.* 256:2843-2850 (1981).
- Axelson, J. and Thesleff, S. A study of super sensitivity in denervated mammalian skeletal muscle. *J. Physiol. Lond.* 149:178-193 (1959).
- Barnard, E.A., Coates, V., Dolly, J.O., and Mallick, B. Binding of  $\alpha$ -Bungarotoxin and cholinergic ligands to acetylcholine receptors in

- the membrane of skeletal muscle. *Cell. Biol. Int. Rep.* 1:99-106 (1977).
- Barnard, E.A., Wieckowski, J., and Chiu, T.H. Cholinergic receptor molecules and cholinesterase molecules at mouse skeletal muscle junctions. *Nature (Lond.)* 234:207-209 (1971).
- Barrantes, F.J. Endogenous chemical receptors: some physical aspects. *Ann. Rev. Biophys. Bioeng.* 8:287-321 (1979).
- Barrett, E.F. and Stevens, C.F. The kinetics of transmitter release at the frog neuromuscular junction. *J. Physiol. Lond.* 227:691-708 (1972).
- Bartels, E., Deal, W., Karlin, A., and Mautner, H. Affinity oxidation of the reduced acetylcholine receptor. *Biochem. Biophys. Acta* 203:568-571 (1970).
- Beame, K.G. A voltage clamp of the effect of two lidocaine derivatives on the time course of the endplate currents. *J. Physiol. Lond.* 258:279-300 (1976).
- Ben-Haim, D., Dreyer, F., and Peper, K. Acetylcholine receptor: Modification of synaptic gating mechanisms with a disulfide bond reducing agent. *Pflugers Arch. Ges. Physiol.* 355:19-26 (1975).
- Ben-Haim, D., Landau, E.M. and Silman, I. The role of the reactive disulfide bond in the function of the acetylcholine receptor at the frog neuromuscular junction. *J. Physiol. Lond.* 234:305-325 (1973).
- Beranek, R., and Vyskocil, F. The action of tubocurarine and atropine on the normal and denervated rat diaphragm. *J. Physiol.* 188:53-66 (1967).
- Berg, D.K. and Hall, Z.W. Fate of  $\alpha$ -Bungarotoxin bound to acetylcholine receptors of normal and denervated muscle. *Science* 184:473-475 (1974).

- Berg, D.K., Kelly, R.B., Sargeant, P.B., Williamson, P. and Hall, Z.  
Binding of  $\alpha$ -Bungarotoxin to acetylcholine receptors in mammalian muscle. Proc. Nat. Acad. Sci. USA 69:147-151 (1972).
- Bisecker, G. Molecular properties of the cholinergic receptor purified from electrophorus electricus. Biochemistry 12:4403-4409 (1973).
- Boulter, J. and Patrick, J. Purification of an acetylcholine receptor from a nonfusing muscle cell line. Biochemistry 16:4900-4908 (1977).
- Bourgeois, J.-P., Popot, J.L., Ryter, A. and Changeux, J.-P.  
Quantitative studies on the localization of cholinergic receptor protein in the normal and denervated electroplaque of electrophorus electricus. J. Cell Biol. 79:200-216 (1978).
- Bradley, R.J., Howell, J.H., Romine, W.O., Carl, G.F., and Kemp, G.E.  
Characterization of the nicotinic acetylcholine receptor from rabbit skeletal muscle and reconstitution in planar phospholipid bilayers. Biochem. Biophys. Res. Comm. 68:577-584 (1976).
- Brookes, J.P. and Hall, Z.W. Acetylcholine receptors in normal and denervated rat diaphragm muscle. I. Purification and interaction with [ $I^{125}$ ]  $\alpha$ -Bungarotoxin. Biochemistry 14:2092-2099 (1975).
- Brookes, J.P. and Hall, Z.W. Acetylcholine receptors in normal and denervated rat diaphragm muscle. 2. Comparison of junctional and extrajunctional receptors. Biochemistry 14:2100-2106 (1975).
- Bulger, J.E. and Hess, G.P. Evidence for separate initiation and inhibitory sites in the regulation of membrane potential of electroplax. I. Kinetic studies with  $\alpha$ -Bungarotoxin. Biochem. Biophys. Res. Comm. 54:677 (1973).

- Burgemeister, W., Catterall, W.A., and Witkop, B. Histronicotoxin enhances agonist induced desensitization of acetylcholine receptor. *Proc. Nat. Acad. Sci. USA* 74:5754-5758 (1977).
- Cartaud, J., Benedetti, E.L., Sobel, A.B., and Changeux, J.-P. A morphological study of the cholinergic receptor protein from torpedo marmorata in its membrane environment and in its detergent extracted purified form. *J. Cell. Sci.* 29:313-337 (1978).
- Cartaud, J., Benedetti, L., Cohen, J.B., Meunier, J.-C., and Changeux, J.P. Presence of a lattice structure in membrane fragments rich in nicotinic receptor protein from the electric organ of torpedo marmorata. *FEBS Letters* 33:109 (1973).
- Chang, C.C. and Huang, M.C. Turnover of junctional and extrajunctional acetylcholine receptors of the rat diaphragm. *Nature* 253:643 (1975).
- Chang, C.C., Lu, S.E., Wang, D.N., and Chuang, S.T. A comparison of effects of various sulfhydryl reagents on neuromuscular transmission. *Eur. J. Pharmacol.* 11:195-203 (1970).
- Chang, C.C. and Lee, C.Y. Electrophysiological study of the neuromuscular blocking action of cobra neurotoxin. *Br. J. Pharm.* 28:172-181 (1966).
- Chang, C.C. and Lee, C.Y. Isolation of neurotoxins from the venom of bungarus multicinctus and their modes of neuromuscular blocking action. *Arch. Int. Pharmacodyn. Ther.* 144:241-257 (1963).
- Changeux, J.P., Benedetti, L., Bourgeois, J.P., Brisson, A., Cartaud, J., Devaux, P., Grunhagen, H., Moreau, M., Popot, J.L., Sobel, A., and Weber, M. Some structural properties of the cholinergic receptor protein in its membrane environment relevant to its

- function as a pharmacological receptor. Cold Springs Harbor Symp. Quant. Biol. 40:211-230 (1976).
- Changeux, J.-P., Kasai, M., and Lee, C.Y. Use of a snake venom toxin to characterize the cholinergic receptor protein. Proc. Nat. Acad. Sci. USA 67:1241-1247 (1970).
- Chiu, T.H., Dolly, J.O., and Barnard, E.A. Solubilization from skeletal muscle of two components that specifically bind  $\alpha$ -Bungarotoxin. Biochem. Biophys. Res. Comm. 51:205 (1973).
- Chiu, T.H., Lapa, A.J., Barnard, E.A., and Albuquerque, E.X. Binding of d-tubocurarine and  $\alpha$ -Bungarotoxin in normal and denervated mouse muscles. Exp. Neurol. 43:399-413 (1974).
- Cohen, J.B. In: Molecular Specialization and Symmetry in Membranes, Ed., A.K. Solomon, pp. 99-128. Harvard Univ. Press, Cambridge (1978).
- Cohen, J.B., Weber, M., and Changeux, J.P. Effects of local anaesthetics and calcium on the interaction of cholinergic ligands with the nicotinic receptor protein from torpedo marmorata. Mol. Pharmacol. 10:904-932 (1974).
- Colquhoun, D. and Rang, H.P. Effects of inhibitors on the binding of iodinated  $\alpha$ -Bungarotoxin to acetylcholine receptors in rat muscle. Mol. Pharm. 12:519-535 (1976).
- Colquhoun, D., Rang, H.P., and Ritchie, J.M. The binding of tetrodotoxin and  $\alpha$ -Bungarotoxin to normal and denervated mammalian muscle. J. Physiol. Lond. 240:199-226 (1974).
- Creese, R. and England, J.M. Decamethonium in depolarized muscle and the effects of tubocurarine. J. Physiol. Lond. 210:345-361 (1971).

- Creese, R. and MacLagen, J. Entry of decamethonium in rat muscle studied by autoradiography. *J. Physiol. Lond.* 210:363-386 (1971).
- Dale, H.H. The action of certain esters and ethers of choline, and their relation to muscarine. *J. Pharmac. Exp. Ther.* 6:147-190 (1914).
- Daly, J.W., Karle, I., Meyers, C.W., Tokuyama, T., Waters, J.A., and Witkop, B. Histrionicotoxins: Roentgen-ray analysis of the novel allenic and spiroalkaloid isolated from a Colombian frog dendrobates histrionicus. *Proc. Nat. Acad. Sci. USA* 68:1870-1875 (1971).
- Daniels, M.P. and Vogel, Z. Immunoperoxidase staining of  $\alpha$ -Bungarotoxin binding sites in muscle endplates shows distribution of acetylcholine receptors. *Nature* 254:339-341 (1975).
- Del Castillo, J. and Katz, B. The membrane change produced by the neuromuscular neurotransmitter. *J. Physiol. Lond.* 125:546-565 (1954).
- Devreotes, P.N. and Fambrough, D.M. Acetylcholine receptor turnover in membranes of developing muscle fibers. *J. Cell Biol.* 65:335 (1975).
- Diamond, J. and Miledi, R. A study of foetal and new-born rat muscle fibres. *J. Physiol.* 162:393-408 (1962).
- Dolly, J.O. Biochemistry of acetylcholine receptor from skeletal muscle. In: *International Reviews of Biochem.*, v. 26, pp. 257-309. Ed., K.F. Tipton. University Park Press, Baltimore (1979).
- Dolly, J.O., Albuquerque, E.X., Sarvey, J.M., Mallick, B., and Barnard, E.A. Binding of perhydro-histrionicotoxin to the postsynaptic membrane of skeletal muscle in relation to its blockade of acetylcholine induced depolarization. *Mol. Pharmacol.* 13:1-14 (1977).

- Dolly, J.O. and Barnard, E.A. Purification and characteristics of an acetylcholine receptor from mammalian skeletal muscle. *Biochemistry* 16:5053-5060 (1977).
- Eccles, J.C., Katz, B., and Kuffler, S.W. Nature of the "endplate potential" in curarized muscle. *J. Neurophysiol.* 4:362-387 (1941).
- Edelstein, S.J., Beyer, W.B., Eldefrawi, A.T., and Eldefrawi, M.E. Molecular weight of the acetylcholine receptors of electric organs and the effect of Triton X-100. *J. Biol. Chem.* 250:6101-6106 (1975).
- Eldefrawi, M.E. and Eldefrawi, A.T. Biochemical studies of the ionic channel of torpedo acetylcholine receptor. *Adv. Cytopharmacol.* 3:213-223 (1979).
- Eldefrawi, A.T., Eldefrawi, M.E., Albuquerque, E.X., Oliveria, A.C., Mansour, N., Adler, M., Daly, J.W., Brown, G.B., Burgermeister, W., and Witkop, B. Perhydrohistrionicotoxin: A potential ligand for the ion conductance modulator of the acetylcholine receptor. *Proc. Nat. Acad. Sci. USA* 74:2172-2176 (1977).
- Eldefrawi, M.E. and Eldefrawi, A.T. Purification and molecular properties of the acetylcholine receptors from torpedo electroplax. *Arch. Biochem. Biophys.* 159:362 (1973).
- Eldefrawi, A.T., Bakry, N.M., Eldefrawi, E.M., Tsai, M.C., and Albuquerque, E.X. Nereistoxin interaction with the acetylcholine receptor ionic channel complex. *Mol. Pharmacol.* 17:172-179 (1980).
- Eldefrawi, M.E., Eldefrawi, A.T., Mansour, N.A., Daly, J.W., Witkop, B., and Albuquerque, E.X. Acetylcholine receptor and ionic channel of torpedo electroplax: binding of perhydrohistrionicotoxin to membrane and solubilized preparations. *Biochemistry* 17:5474-5484 (1978).

- Elliot, J. and Raftery, M. Interactions of perhydrohistrionicotoxin with the postsynaptic membranes. *Biochem. Biophys. Res. Comm.* 77:1347-1353 (1977).
- Fambrough, D.M. and Hartzell, H.C. Acetylcholine receptors: Number and distribution at neuromuscular junctions in rat diaphragm. *Science* 176:189-191 (1972).
- Fatt, P. and Katz, B. An analysis of the endplate potential reduced with an intracellular electrode. *J. Physiol. Lond.* 115: 320-370 (1951).
- Feldberg, W. and Fressard, A. The cholinergic nature of the nerves to the electric organ of torpedo (*torpedo varmorata*). *J. Physiol. Lond.* 101:200-216 (1942).
- Fertuck, H.C. and Salpeter, M.M. Quantitation of junctional and extrajunctional acetylcholine receptors by electron microscope autoradiography after [<sup>125</sup>I]- $\alpha$ -Bungarotoxin binding at mouse neuromuscular junctions. *J. Cell. Biol.* 69:144-158 (1976).
- Fertuck, H.C. and Salpeter, M.M. Localization of acetylcholine receptors by [<sup>125</sup>I]-labeled  $\alpha$ -Bungarotoxin binding at mouse motor endplate. *Proc. Nat. Acad. Sci. USA* 71:1376-1378 (1974).
- Fleisch, J.H., Krzan, M.C., and Titus, E. Alterations in pharmacological receptor activity by dithiothreitol. *Am. J. Physiol.* 227:1243-1248 (1974).
- Franklin, G.I. and Potter, L.T. Studies of the binding of  $\alpha$ -Bungarotoxin to membrane-bound and detergent-dispersed acetylcholine receptors from torpedo electric tissue. *FEBS Letts.* 28:101-106 (1972).

- Froehner, S.C., Karlin, A., and Hall, Z.W. Affinity alkylation labels two subunits of the reduced acetylcholine receptor from mammalian muscle. *Proc. Nat. Acad. Sci. USA* 74:4685-4688 (1977a).
- Froehner, S.C., Reiness, C.A., and Hall, Z.W. Subunit structure of the acetylcholine receptor from denervated rat skeletal muscle. *J. Biol. Chem.* 252:8589-8596 (1977b).
- Fu, J.L., Donner, D.B., and Hess, G.P. Half-of-the-sites reactivity of the membrane-bound electrophorus electricus acetylcholine receptors. *Biochem. Biophys. Res. Comm.* 60:1072 (1974).
- Fulpius, B.W., Klett, R.P., Cooper, D., and Reich, E. The nicotinic acetylcholine receptor: Characteristics of a molecule isolated from electrophorus electricus. *Proc. 5th Int. Cong. Pharmacol.* 5:68-80 (1973).
- Furukawa, T. and Furukawa, A. Effects of methyl and ethyl derivatives of ammonium on the neuromuscular junction. *J. Physiol.* 9:130-147 (1959).
- Gage, P.W., McBurney, R.N., and Schneider, T. Effect of some aliphatic alcohols on the conductance change caused by a quantum of acetylcholine at the toad endplate. *J. Physiol.* 244:409-429 (1975).
- Gage, P.W. and Armstrong, C.M. Miniature endplate currents in voltage clamp muscle fibers. *Nature* 218:363-365 (1968).
- Gage, P.W. and McBurney, R.N. Some factors influencing the amplitude and time course of miniature endplate potentials in toad sartorius. *Proc. Australian Physiol. Pharmacol. Soc.* 2:41 (1971).
- Gage, P.W., McBurney, R.N., and Van Helden, D. Endplate currents are shortened by octanol: Possible role of membrane lipid. *Life Sci.* 14:2277-2283 (1974).

- Grunhagen, H. and Changeux, J.-P. Studies on the electrogenic action of acetylcholine with torpedo marmorata electric organ. IV. Quinacrine: A fluorescent for the conformational transitions of the cholinergic receptor protein. *J. Mol. Biol.* 106:497-535 (1976).
- Hartzell, H.C. and Fambrough, D.M. Acetylcholine receptors. Distribution and extrajunctional density in rat diaphragm after denervation correlated with acetylcholine sensitivity. *J. Gen. Physiol.* 60:248-262 (1972).
- Hartzell, H.C. and Fambrough, D.M. Acetylcholine receptor production and incorporation into membranes of developing muscle fibers. *Dev. Biol.* 30:153-165 (1973).
- Hartzell, H.C., Kuffler, S.W., and Yoshikami, D. The number of ACh molecules in a quantum and the interaction between quanta at the subsynaptic membrane. *Cold Spring Harbor Symp. Quant. Biol.* 40:175-186 (1976).
- Heidmann, T. and Changeux, J.-P. Structural and functional properties of the acetylcholine receptor protein in its purified and membrane bound states. *Ann. Rev. Biochem.* 47:371-411 (1978).
- Heidmann, T., Sobel, A., Popot, J.-L., and Changeux, J.-P. Reconstitution of a functional acetylcholine receptor-conservation of allosteric transitions and recovery of the permeability response: Role of lipids. *Eur. J. Biochem.* 110:35-55 (1980).
- Hess, G.P., Bulger, J.E., Fu, J.L., Hindy, E.F., and Silberstein. Allosteric interactions of the membrane-bound acetylcholine receptor; kinetic studies with  $\alpha$ -Bungarotoxin. *Biochem. Biophys. Res. Comm.* 64:1018-1026 (1975).

- Heuser, J.E., Reese, T.S., and Landis, D.M.D. Functional changes in frog neuromuscular junctions studied with freeze fracture. *J. Neurocytol.* 3:109-131 (1974).
- Horn, R., Brodwick, M.S., and Dickey, W.D. Asymmetry of the acetylcholine channel revealed by quaternary anaesthetics. *Science* 210:205-207 (1980).
- Hucho, F. and Changeux, J.-P. Molecular weight and quaternary structure of the cholinergic receptor protein extracted by detergents from electrophorus electric tissue. *FEBS Lett.* 38:11-15 (1973).
- Hunt, C.C. The adrenergic blocking effect of certain  $\beta$ -chloroethyl amines. *J. Pharmacol. Exp. Therap.* 95:177-184 (1948).
- Karlin, A. Chemical modification of the active site of the acetylcholine receptor. *J. Gen. Physiol.* 54:2458-2645 (1969).
- Karlin, A. and Bartels, E. Effects of blocking sulfhydryl groups on the acetylcholine activated permeability system of the electroplax. *Biochem. Biophys. Acta* 126:525-535 (1966).
- Karlin, A. and Winnick, M. Reduction and alkylation of the receptor for acetylcholine. *Proc. Nat. Acad. Sci. USA* 60:668-674 (1968).
- Karlin, A., Weil, C.Z., McNamee, M.G., and Valderrama, R. Facets of the structures of acetylcholine receptors from electrophorus and torpedo. *Cold Springs Harbor Symp. Quant. Biol.* 40:203-210 (1976).
- Kasai, M. and Changeux, J.-P. In vitro excitation of purified membrane fragments by cholinergic agonists. I. Pharmacological properties of the excitable membrane fragments. *J. Memb. Biol.* 6:1-23 (1971).
- Kato, G. and Changeux, J.-P. Studies of the effect of histrionicotoxin on the monocellular electroplax from electrophorus and on the binding of [ $^3$ H] acetylcholine to membrane fragments from torpedo

- marmorata. Mol. Pharmacol. 12:92-100 (1976).
- Katz, B. and Miledi, R. Spontaneous and evoked activity of motor nerve endings in calcium Ringer. J. Physiol. Lond. 203:689-706 (1970).
- Katz, B. and Miledi, R. The statistical nature of the acetylcholine potential and its molecular components. J. Physiol. Lond. 224:665-699 (1972).
- Katz, B. and Miledi, R. The characteristics of "endplate noise" produced by different depolarizing drugs. J. Physiol. Lond. 230:707-717 (1973).
- Katz, B. and Miledi, R. The effect of  $\alpha$ -Bungarotoxin on acetylcholine receptors. Brit. J. Pharmacol. 49:138-139 (1973).
- Kemp, G., Dolly, J.O., Barnard, E.A., and Wenner, C.E. Reconstitution of a partially purified endplate acetylcholine receptor preparation in lipid bilayer membranes. Biochem. Biophys. Res. Comm. 55:1044-1050 (1973).
- Kemp, G., Morley, B., Dwyer, D., and Bradly, R.L. Purification and characterization of nicotinic acetylcholine receptors from muscle. Membrane Biochem. 3:229-257 (1980).
- Keynes, R.D. and Martinez-Ferreira, H. Membrane potentials in the electroplates of the electric eel. J. Physiol. Lond. 119:315-351 (1953).
- Klett, R.P., Fulpius, B.W., Cooper, D., Smith, M., Reich, E., and Possani, D. The acetylcholine receptor. I. Purification and characterization of a macromolecule isolated from electrophorus electricus. J. Biol. Chem. 248:6841-6853 (1973).

- Kloog, Y., Gabriealevitz, A., Kalir, A., Balderman, D., and Sokolovsky, M. Functional evidence for a second binding site of nicotinic antagonists using phencyclidine derivatives. *Biochem. Pharmacol.* 28:1447-1450 (1979).
- Koketsu, K. and Nishi, S. Restoration of neuromuscular transmission in sodium free hydrazinium solution. *J. Physiol. Lond.* 147:239-252 (1959).
- Kordas, M. The effect of membrane polarization on the time course of the endplate current in frog sartorius muscle. *J. Physiol.* 204:493 (1969).
- Kordas, M. An attempt at an analysis of the factors determining the endplate current. II. Temperature. *J. Physiol.* 224:333 (1972).
- Krodel, E.K., Beckman, R.A., and Cohen, J.B. Identification of the local anaesthetic binding site from torpedo marmorata electric tissue. *Mol. Pharm.* 15:294-312 (1979).
- Kuffler, S.W. and Yoshikami, D. The distribution of acetylcholine sensitivity at the post synaptic membrane of vertebrate skeletal twitch muscles: Iontophoretic mapping in the micron range. *J. Physiol.* 244:703-730 (1975).
- Langley, J.N. On the contraction of muscle, chiefly in relation to the presence of "receptor substances." Part I. *J. Physiol.* 36:347-384 (1907).
- Lapa, A.J., Albuquerque, E.X., Sarvey, J.M., Daly, J., and Witkop, B. Effects of histrionicotoxin on the chemosensitive and electrical properties of skeletal muscle. *Exper. Neurol.* 47:558-580 (1975).
- Lee, C.Y. Chemistry and pharmacology of polypeptide toxins in snake venoms. *Ann. Rev. Pharmacol.* 12:265-287 (1972).

- Lee, C.Y. and Tseng, L.F. Distribution of bungarus multicinctus venom following envenomation. *Toxicol* 3:281-290 (1966).
- Lee, C.Y., Tseng, L.F., and Chiu, T.H. Influence of denervation on localization of neurotoxins from elapid venoms in rat diaphragm. *Nature* 215:1177-1178 (1967).
- Lindstrom, J. and Patrick, J. In: *Synaptic Transmission and Neuronal Interaction*. (M.V.L. Bennet, Ed.), Raven Press, New York, p. 191 (1974)
- Lindstrom, J.M., Singer, S., and Lennox, E.S. The effects of reducing and alkylating agents on the acetylcholine receptor activity of frog sartorius muscle. *J. Memb. Biol.* 11:217-226 (1973).
- Lindstrom, J., Walter, B., and Einarson, B. Immunochemical similarities. Subunits of acetylcholine receptors from torpedo electrophorus and mammalian muscle. *Biochemistry* 18:4471-4479 (1979).
- Magleby, K.L. and Stevens, C.F. The effect of voltage on the time course of endplate currents. *J. Physiol.* 223:151 (1972).
- Magleby, K.L. and Stevens, C.F. A quantitative description of end plate currents. *J. Physiol.* 223:173 (1972).
- Manthey, A.A. The effect of calcium on the desensitization of membrane receptors at the neuromuscular junction. *J. Gen. Physiol.* 49:963-976 (1966).
- Martinez-Carrion, M., Sator, V., and Raftery, M.A. The molecular weight of an acetylcholine receptor isolated from torpedo californica. *Biochem. Biophys. Res. Comm.* 65:129-137 (1975).
- Masukawa, L. and Albuquerque, E.X. Voltage- and time-dependent action of histrionicotoxin on the end plate current of frog muscle. *J. Gen. Physiol.* 72:351-368 (1978).

- Merlie, J.P., Changeux, J.-P. and Gros, F. Skeletal muscle acetylcholine receptor. Purification, characterization and turnover in muscle cell cultures. *J. Biol. Chem.* 253:2882-2891 (1978).
- Meunier, J.-C., Sealock, R., Olsen, R., and Changeux, J.P. Purification and properties of the cholinergic receptor proteins from *electrophorus electricus* electric tissue. *Eur. J. Biochem.* 45:371-394 (1974).
- Michaelson, D.M. and Raftery, M.A. Purified acetylcholine receptor: its reconstitution to a chemically excitable membrane. *Proc. Nat. Acad. Sci. USA* 71:4768-4772 (1974).
- Miledi, R. The acetylcholine sensitivity of frog muscle fibers after complete or partial denervation. *J. Physiol. Lond.* 151:1-23 (1960).
- Miledi, R., Molinoff, P., and Potter, L.T. Isolation of the cholinergic receptor protein of torpedo electric tissue. *Nature Lond.* 229:554-557 (1971).
- Miledi, R. and Potter, L.T. Acetylcholine receptors in muscle fibers. *Nature* 233:599-603 (1971).
- Mittag, T.W. and Tormay, A. Disulfide bonds in nicotinic receptors. *Fed. Proc.* 21:547 (1970) abs.
- Mittag, T.W., Kornfeld, P.K., Tormay, A., and Woo, C. Detection of anti-acetylcholine receptor factors in serum and thymus from patients with myasthenia gravis. *N. Eng. J. Med.* 294:691-694 (1976).
- Mittag, T.W., Tormay, A., and Massa, T. Heterogeneity of acetylcholine receptors in denervated muscle: Interactions of receptor with immunoglobulin from patients with myasthenia gravis. *Mol. Pharm.* 14:60-68 (1978).

- Moore, H.-P.H., Hartig, P.R., Wu, W. C.-S., and Raftery, M.A. Rapid cation flux from torpedo californica membrane vesicles: Comparison of acetylcholine receptor enriched and selectively extracted vesicles. *Biochem. Biophys. Res. Comm.* 88:735-743 (1979).
- Nastuk, W.L. Some ionic factors that influence the action of acetylcholine at the muscle endplate membrane. *Ann. N.Y. Acad. Sci.* 81:317-327 (1959).
- Nathanson, N.M. and Hall, Z.W. In situ labeling of torpedo and rat muscle acetylcholine receptor by a photoaffinity derivative of  $\alpha$ -Bungarotoxin. *J. Biol. Chem.* 255:1698-1703 (1980).
- Nathanson, N. and Hall, Z. Subunit structure and peptide mapping of junctional and extrajunctional AChR from rat muscle. *Biochemistry* 18:3392-3399 (1979).
- Neher, E. and Sakman, B. Noise analysis of drug induced voltage clamp currents in denervated frog muscle fibres. *J. Physiol. Lond.* 258:705-730 (1976).
- Neher, E., and Steinbach, J.H. Local anaesthetics transiently block currents through single acetylcholine receptor channels. *J. Physiol.* 277:153-176 (1978).
- Neher, E. and Stevens, C.F. Conductance fluctuations and ionic pores in membranes. *Ann. Rev. Biophys. Bioeng.* 6:345-381 (1977).
- Neubig, R.R., Krodel, E.K., Boyd, N.D., and Cohen, J.B. Acetylcholine and local anaesthetic binding to torpedo nicotinic postsynaptic membranes after removal of non-receptor peptides. *Proc. Nat. Acad. Sci. USA* 76:640-694 (1979).
- Nickel, E. and Potter, L.T. Ultrastructure of isolated membranes of torpedo electric tissue. *Brain Res.* 57:508 (1973).

- Patrick, J. and Lindstrom, J. Autoimmune response to acetylcholine receptor. *Science* 180:871-872 (1973).
- Porter, C.W., Chiu, T.N., Wieckowski, J., and Barnard, E.A. Types and locations of cholinergic receptor-like molecules in muscle fibres. *Nature New Biol.* 241:3-7 (1973).
- Porter, C.W., Barnard, E.A., and Chiu, T.N. The ultrastructural localization and quantitation of cholinergic receptors at the mouse motor endplate. *J. Memb. Biol.* 14:383-402 (1973).
- Rafferty, M.A., Vandlin, R.L., Reed, K.L., and Lee, T. Characterization of torpedo californica acetylcholine receptor: its subunit composition and ligand-binding properties. *Cold Spring Harbor Symp. Quant. Biol.* 40:193-202 (1976).
- Rafferty, M.A., Schmidt, J., and Clark, D.G. Specificity of  $\alpha$ -Bungarotoxin binding to torpedo californica electroplax. *Arch. Biochem. Biophys.* 152:882-886 (1972).
- Rafferty, M.A., Schmidt, J., Clark, D.G., and Calcott, R.G. Demonstration of a specific  $\alpha$ -Bungarotoxin binding component in electrophorus electricus electroplax membranes. *Biochem. Biophys. Res. Comm.* 45:1622-1629 (1971).
- Rafto, S. and Froehner, S.C. Comparison of the subunits of torpedo californica acetylcholine receptor by peptide mapping. *Biochemistry* 18:301-307 (1979).
- Rang, H.P. Acetylcholine receptors. *Quart. Rev. Biophys.* 7:283-399 (1975).
- Rang, H.P. and Ritter, J.M. The effect of disulfide bond reduction on the properties of cholinergic receptors in chick muscle. *Mol. Pharm.* 7:620-631 (1971).

- Rosen, G.M., Ehrenpreis, S., Mittag, T.W., and Stubbins, J.F. Physical and pharmacologic properties of an irreversible local anesthetic. *J. Med. Chem.* 14:514-516 (1971).
- Rosen, G.M., Ehrenpreis, S., and Karoutsou, A. Neuromuscular blocking activity of a series of  $\beta$ -haloethylamines. *Arch. Int. Pharmacodyn.* 204:242-251 (1973).
- Ross, D.H. and Triggle, D.J. Further differentiation of cholinergic receptors in leech muscle. *Biochem. Pharmac.* 21:2533-2536 (1972).
- Ross, J.B.A., Klymkowsky, M.W., Agard, D.A., and Stroud, R.M. Structural studies of a membrane-bound acetylcholine receptor from torpedo californica. *J. Mol. Biol.* 116:635-659 (1977).
- Ruff, R.L. A quantitative analysis of local anaesthetic alteration of miniature end plate currents and end plate current fluctuations. *J. Physiol. Lond.* 264:89-124 (1977).
- Ruiz-Manresa, F. and Grundfest, H. Synaptic electrogenesis in eel electroplax. *J. Gen. Physiol.* 57:71-92 (1971).
- Salpeter, M.M. and Eldefrawi, M.E. Size of endplate compartment densities of acetylcholine receptor and other quantitative aspects of neuromuscular transmission. *Cytochem.* 21:769-778 (1973).
- Sarvey, J.M., Albuquerque, E.X., Eldefrawi, A.T., and Eldefrawi, M. Effects of  $\alpha$ -Bungarotoxin and reversible cholinergic ligands on normal and denervated mammalian skeletal muscle. *Membrane Biochem.* 1:131-157 (1978).
- Schimerlik, M.I., Quast, U., and Raftery, M.A. Ligand induced changes in membrane-bound acetylcholine receptor observed by ethidium fluorescence. 3. Stopped-flow studies with histrionicotoxin. *Biochemistry* 18:1902-1906 (1979).

- Shorr, R.G., Dolly, J.O., and Barnard, E.A. Composition of acetylcholine receptor protein from skeletal muscle. *Nature Lond.* 274:283-284 (1978).
- Silman, I. and Karlin, A. Acetylcholine receptor: Covalent attachment of depolarizing groups at the active site. *Science* 164:1420-1421 (1969)
- Sobel, A., Heidmann, T., Hofler, J., and Changeux, J.-P. Distinct protein components for the acetylcholine receptor site and the binding site for local anaesthetics and histrionicotoxin. *Proc. Nat. Acad. Sci. USA* 75:510-514 (1978).
- Sobel, A., Heidmann, T., Cartaud, J., and Changeux, J.-P. Reconstitution of a functional acetylcholine receptor - polypeptide chains, ultrastructure, and binding sites for acetylcholine and local anaesthetics. *Eur. J. Biochem.* 110:13-33 (1980).
- Steinbach, A.B. Alteration by xylocaine (lidocaine) and its derivatives of the time course of the end plate potential. *J. Gen. Physiol.* 52:144-161 (1968).
- Stevens, C.F. Molecular basis for postjunctional conductance increases induced by acetylcholine. *Cold Spring Harbor Symp. Quant. Biol.* 40:169-173 (1975).
- Stevens, C.F. In: *Synaptic Transmission and Neuronal Interactions.* (M.V.L. Bennett, ed.) Raven Press, New York, pp. 45-58 (1974).
- Su, C., Chang, C.C., and Lee, C.Y. In: *Animal Toxins*, pp. 257-267. Ed., F.E. Russell and P.R. Saunders, Pergamon, Oxford and New York (1976).

- Sytkowski, A.J., Vogel, Z., and Nirenberg, M.W. Development of acetylcholine receptor clusters on cultured muscle cells. *Proc. Nat. Acad. Sci. USA* 70:270-274 (1973).
- Takeuchi, N. Effects of calcium on the conductance change of the end plate membrane during the action of transmitter. *J. Physiol. Lond.* 167:141-155 (1963).
- Takeuchi, A. and Takeuchi, N. Active phase of frog's end plate potential. *J. Neurophysiol.* 22:295-305 (1959).
- Tiedt, T.N., Mansour, N., Albuquerque, E.X., Eldefrawi, M.E., and Triggle, D.J. Diphenylacetate derivative on the end plate currents of frog sartorius muscle. *Fed. Proc.* 37:787 (1978).
- Tsai, M.-C., Aronstam, R.S., Eldefrawi, M.E., Eldefrawi, A.T., and Albuquerque, E.X. Phencyclidine interaction with the nicotinic acetylcholine receptor ion channel complex and effect on potassium conductance. *Fed. Proc.* 38:274 (1979).
- Tsai, M.-C., Oliveira, A.C., Albuquerque, E.X., Eldefrawi, M.E., and Eldefrawi, A.T. Mode of action of quinacrine on the acetylcholine receptor ion channel complex. *Mol. Pharmacol.* 16:909-921 (1979).
- Tsai, M.-C., Mansour, A., Eldefrawi, A.T., Eldefrawi, M.E., and Albuquerque, E.X. Mechanism of action of amantadine on neuromuscular transmission. *Mol. Pharmacol.* 14:787-803 (1978).
- Vincent, A. Immunology of acetylcholine receptors in relation to myasthenia gravis. *Physiol. Rev.* 60:756-824 (1980).
- Vogel, Z., Sytkowski, A.J., and Nirenberg, M.W. Acetylcholine receptors of muscle growth in vitro. *Proc. Nat. Acad. Sci. USA* 69:135-146 (1972).