HETEROGENEITY OF THE NICOTINIC ACETYLCHOLINE RECEPTOR EXTRACTED FROM DENERVATED RAT HINDLIMB MUSCLE

by

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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.

1978

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.

December 12, 1978
Date

Chairman of Examining Committee

December 12, 1978
Date

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Abstract

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The interactions of the extrajunctional nicotinic acetylcholine receptor, extracted from denervated rat hindlimb muscle, with α-bungarotoxin (α-BuTx), Concanavalin-A (Con-A) and immunoglobulins isolated from the sera of patients with myasthenia gravis have been studied. Investigation of the receptor-toxin interaction using kinetic methods showed two distinct toxin bidning sites. The association of toxin to hydroxylapatite prepared AChR did not follow simple second-order kinetics but was best described in terms of two classes of toxin binding sites with rate constants of $8.23 \times 10^4\ M^{-1}s^{-1}$ and $0.35 \times 10^4\ M^{-1}s^{-1}$. Dissociation of toxin-receptor complexes occurred in a biphasic manner with a rapidly dissociating component ($k_{-1} = 2.49 \times 10^{-5}s^{-1}$) and a slowly dissociating component ($k_{-2} = 2.43 \times 10^{-6}s^{-1}$). Furthermore, computer analysis of the kinetics data showed these two kinetically distinct toxin-binding sites to be present in equal amounts.

Incubation of these receptors with soluble Con-A prior to labelling with α-BuTx decreases the total binding capacity by approx-

imately 50%. Kinetic studies indicate that the Con-A induced inhibition of toxin binding appears restricted to those receptors with fast rates of association with α-BuTx.

Kinetic analysis of the toxin-receptor interaction using receptors further purified using Lens culinaris-Agarose (LCA) affinity chromatography indicated that the two kinetically distinct toxin binding sites were separable and present in equal amounts. The receptors readily eluted from the LCA column exhibited fast rates of association and dissociation with α-BuTx and represented 50% of the total receptor population. Those receptors which remained tightly bound to the LCA column were assumed to exhibit slow rates of association and dissociation with α-BuTx. Incubation of LCA-purified AChR with soluble Con-A resulted in 100% inhibition of toxin binding.

The two classes of toxin binding sites were further subdivided based on interactions of receptor with immunoglobulin G fractions from sera of patients with myasthenia gravis. Three distinct antibody types were found in these sera. The first caused a 50% maximum inhibition of hydroxylapatite and LCA prepared receptor binding to Con-A-Sepharose affinity columns when the receptors were treated with myasthnic IgG prior to labelling with α-BuTx. This indicates that both types of toxin binding sites (fast and slow) are further divided into two separable subclasses. A second antibody, found in only a small number of myasthenics, caused a 30% maximum inhibition of toxin binding to hydroxylapatite receptors, but had no effect on toxin binding to LCA purified receptors, indicating that this antibody inhibited toxin binding to a subclass of those receptors with slow kinetics of toxin binding. Furthermore, the inhibition of

α-BuTx binding to receptors pre-treated with this antibody is additive with the Con-A induced inhibition of toxin binding, indicating that the toxin-blocking antibody affects a subpopulation of receptors different from those affected by Con-A. A third antibody bound to all subclasses of receptors without altering the kinetics of toxin binding to the receptor nor the interaction of the receptor with soluble or Sepharose bound Con-A. Its sole effect was to immunoprecipitate AChR when the antibody-AChR-toxin complex was exposed to anti-human IgG.

In summary, the present study shows that the solubilized extrajunctional nicotinic acetylcholine receptor extracted from denervated rat hindlimb muscle consists of four subclasses of toxin binding sites, which appear to be seaparate molecular entities, based on their interaction with α-BuTx, Con-A and myasthenic anti-receptor antibodies. The biological significance of this finding remains to be elucidated.

DEDICATION

To my parents, Gaetano and Mary Massa, for their love, support and countless sacrifices.

ACKNOWLEDGEMENT

The author would like to thank several individuals for their assistance which made this work possible:

Dr. Dan Edwards, for his assistance with the computer analysis of the data.

Dr. L. Allen Barker, for his helpful criticism during the course of this study and editing of the final manuscript.

Drs. Joseph Goldfarb, Adam Bender and Tatsuji Namba for their valuable suggestions during preparation of the final manuscript.

Ms. Anne Tormay, for her expert technical advice.

Ms. Joan Bratton, for her help in coordinating acquisition of the serum specimens.

Vicki and Paavo Zakin, who typed and proof read the final manuscript.

Special thanks are due to my thesis advisor, Dr. Tom Mittag, who was always available to listen and offer constructive criticism throughout the course of this study. It is difficult to say simply thank you to someone who has been not only my advisor, but also a good friend. I will always value our relationship.

No acknowledgement would be complete without mention of my wife's love, understanding and tolerance during my graduate studies. Without her encouragement I could not have completed this work.

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I. INTRODUCTION

A. HISTORICAL BACKGROUND

A large portion of present day basic pharmacological research is devoted to the study of the molecular mechanisms of drug action. Whatever the ultimate effect a drug produces in a biological system, its initiator is a physicochemical interaction between that drug and some functionally important molecule(s) in the organism which has been termed a receptor. Much of the evolution of the concept of receptors and receptor theory parallels research concerning the hypothesis of chemical transmission at synapses and acetylcholine receptors. This association has been maintained to the present day when receptors are being purified and intensively studied.

Although it was not Claude Bernard who originated the idea of receptors, it was his classical experiments with curare that created the basis for this concept. Bernard showed that injection of curare produced paralysis in frog leg muscle and that a curare-poisoned leg could not be stimulated electrically via its nerve, but was sensitive to direct electrical stimulation. The site of action of curare, therefore, was somewhere in the area of contact between nerve and muscle (Bernard, 1857).

Langley (1905, 1906 and 1907) provided evidence for both the chemical hypothesis of neuromuscular transmission and the existence of receptors. By applying nicotine to frog sartorious muscle, he discovered that contraction occured only when nicotine was applied where the nerve entered the muscle fibers. Application to other regions of the muscle had no effect. When curare was applied to the neural region of the muscle it blocked the responses to nerve stimu-

lation and to nicotine, but did not inhibit contraction of the muscle to direct stimulation. Application of nicotine and curare to the nerve had no effect on contraction and furthermore the effects of curare and nicotine were seen even after denervation, implying that their site of action was the muscle. Langley proposed that in each cell (muscle, gland, or nerve) two substances must be distinguished. One, the chief substance, performs the main function of the cell (contraction, secretion or generation of electrical impulses), while the other accessory substance has the task of receiving the action of the nerve and transmitting it to the chief substance. Langley called this accessory substance the "receptive substance".

Independent of the development of the concept of chemical transmission, Paul Erlich's studies on the specificity of the antigen-antibody reaction and chemotherapy (1913) also contributed to the concept of receptors. Erlich envisioned receptors as "side chains" which were present on all cells and essential for their life processes. Antibodies and drugs combine specifically with the "side chains" (e.g., sulfhydryl or amino groups) of invading organisms but not those of the host. Only drugs or antibodies of the correct shape and chemical composition could effectively combine with a parasite's receptors and any slight modification of the drug or antibody affected their potency by altering their ability to combine with the receptor.

In 1921, Loewi demonstrated that a chemical substance could be released from a nerve upon stimulation. He found that stimulation of the vagus nerve innervating frog heart muscle released a chemical ("Vagusstoff"), later shown to be acetylcholine (Loewi and Navratil,

1926), which inhibited another isolated frog heart when added to the perfusate. This confirmed the earlier classical experiments of Sir Henry Dale (1914), which showed that acetylcholine could affect heart rate even after denervation by sectioning both vagus nerves. Furthermore, Dale established that a given transmitter could have more than one specific receptor site with which it interacts. He observed a "muscarinic" action of several choline derivatives which mimicked the effects of parasympathetic stimulation and the alkaloid muscarine in isolated heart preparations and a "nicotinic" action of nicotine and quaternary ammonium ions exerted at skeletal muscles and autonomic ganglia. Dale (1914) demonstrated that this series of choline esters exhibited a spectrum of these two actions, ranging from purely muscarinic to purely nicotinic. The muscarinic actions were abolished by atropine without affecting the nicotinic effects of a particular compound. Acetylcholine was found to possess both nicotinic and muscarinic actions which were inhibited by curare and atropine, respectively (Dale, 1914).

Dale and his associates expanded their studies of chemical transmission at the neuromuscular junction and developed the criteria that are still used to establish whether a substance is a transmitter (see Paton, 1958). Feldberg (1943) deomonstrated the presence and synthesis of acetylcholine at the neuromuscular junction as well as its release from the nerve terminal upon stimulation of the nerve (Dale, Feldberg and Vogt, 1936). Finally, it was demonstrated that the muscle showed the same response to acetylcholine regardless of whether direct nerve stimulation or artificial application was used (Brown, Dale and Feldberg, 1936; Bacq and Brown, 1937; Brown,

1937). In the 1950's it was established that acetylcholine initiated muscle action potentials and contraction by causing increased permeability to Na^+ and K^+ in the endplate region of the postsynaptic membrane (Fatt and Katz, 1952; Del Castillo and Katz, 1956; Takeuchi and Takeuchi, 1960). Nachmanshon (1955) proposed that the acetylcholine receptor at the endplate of the neuromuscular junction was a protein and that the binding of acetylcholine to this protein initiated a conformational change at the endplate which subsequently opens ion channels resulting in the increased Na^+ and K^+ permeability.

Quantification of the drug-receptor interaction has also been of great interest to pharmacologists. A.J. Clark (1933) provided the first mathematical model for the interaction of a drug with its receptor. Clark found that only a very small fraction of the total available cell surface area was actually occupied by a drug in producing its effect, and suggested that the area occupied by the drug might correspond to the "receptors" described by Langley and Erlich. Clark assumed that the interaction of a drug with a receptor was analogous to the adsorption of molecules on the surface of a catalyst as described by Langmuir (1916, 1918). He further suggested that drug activity (i.e., the response of a system to the drug) was a function of its affinity constant or its ability to adsorb to the receptors. This proposal led to the "occupation" theory of drug-receptor interaction which was later modified by Ariens (1954) and Stephenson (1956). Paton (1961) proposed that the rate of combination of the drug with its receptor was the important parameter of drug-receptor interaction ("rate" theory). There has been much debate

regarding the mathematical models of drug-receptor interaction and as yet no one model appears sufficient to adequately describe the events of ligand-receptor recognition and/or subsequent response.

Thus, despite the wealth of physiological and pharmacological research on neuromuscular transmission, all that was known about the acetylcholine receptor until the mid-1960's was that it should exist on the endplate region of the postjunctional membrane and was somehow involved in the membrane changes responsible for depolarization. Little was known regarding the actual binding of acetylcholine to its "receptor" and how this interaction resulted in depolarization of the endplate. The discovery of α-bungarotoxin, a highly specific ligand for the acetylcholine receptor (AChR) (Chang, 1960; Chang and Lee, 1963), and the isolation of highly purified receptor preparations (Changeux et al., 1970) have led to a great acceleration in receptor research (see reviews by Karlin, 1974, 1977; Cohen and Changeux, 1975; Rang, 1975; Eldefrawi and Eldefrawi, 1977) concerning the molecular structure of the receptor, its organization in the postsynaptic membrane and the events following recognition of acetylcholine which eventually result in membrane depolarization.

Two more recent developments have also been utilized to study the molecular biology of the nicotinic acetylcholine receptor. The first is the discovery that the receptor is a glycoprotein capable of binding Concanavalin-A (Brockes and Hall, 1975a; Mattson and Heilbronn, 1975; Almon and Appel, 1976a), a lectin which binds specifically to α-D-mannose and α-D-glucose and their glycosides (reviewed by Sharon and Lis, 1972). The other is the finding that antibodies raised to acetylcholine receptors interfere with neuromuscular trans-

mission and produce symptoms in animals that closely resemble the neuromuscular disorder myasthenia gravis (Patrick and Lindstrom, 1973). Similar antibodies have subsequently been found in the sera of patients with mysthenia gravis. This finding has contributed significantly to the understanding of the pathogenesis of the apparently autoimmune disorder as well as the study of the acetylcholine receptor.

B. USE OF A α-BUNGAROTOXIN AS A LIGAND FOR THE NICOTINIC ACETYLCHOLINE RECEPTOR

The venoms of snakes belonging to the Elapidae family (cobras and kraits) are extemely toxic and produce flaccid paralysis and respiratory failure in animals. These effects are attributed to the neurotoxins contained in these venoms (Meldrum, 1965; Jimenez-Parras, 1968; Lee, 1970, 1971; Simpson, 1974; Tu, 1977). Venom of the banded krait, Bungarus multicinctus, contains four neurotoxins capable of blocking neuromuscular transmission (Chang and Lee, 1963). α-Bungarotoxin produces a virtually irreversible, non-depolarizing, curare-like block of the neuromuscular junction by binding to the postjunctional membrane. γ- and β-bungarotoxin block transmission by inhibiting the release of acetylcholine from the motor nerve terminals (Chang and Lee, 1963). Another venom component has acetylcholinesterase activity. The biological activity of a α-bungarotoxin (α-BuTx) makes it an extremely useful tool in the study of the nicotinic acetylcholine receptor.

α-Bungarotoxin is a basic protein of 8000 daltons, consisting of a single peptide with 74 amino acids cross-linked by 5 disulfide bridges (Mebs et al., 1971; Chicheportiche et al., 1975). The amino acid sequence and primary structure of α-BuTx is very similar to that of the cobra α-neurotoxins (Chicheportiche et al., 1975; Tu, 1977). The integrity of the disulfide bonds in the α-neurotoxins is essential, as their reduction results in complete loss of toxicity (Lee et al., 1960; Yang, 1967). The neuromuscular blocking action of the cobra α-neurotoxins is slowly reversible (Su et al., 1967; Tu, 1977). In contrast to cobra α-neurotoxin, the block produced by

α-BuTx is much less reversible (Chang and Lee, 1963; Lee and Chang, 1966; Changeux et al., 1970; Miledi et al., 1971; Tu, 1977). This is thought to be due to the greater content of hydrophobic amino acid residues, such as valine, alanine and phenylalanine, in α-BuTx than in other α-neurotoxins.

α-Bungarotoxin, as well as cobra α-neurotoxins, blocks neuromuscular transmission in a non-depolarizing manner very similar to that produced by curare (Chang and Lee, 1963; Lee and Chang, 1966; Changeux et al., 1970; Miledi et al., 1971). These toxins, as well as curare, act at specific sites on the postjunctional membrane of the neuromuscular junction to block synaptic transmission (Meldrum, 1965; Lee, 1970, 1971). Like curare, α-BuTx inhibits the response of acetylcholine in frog rectus abdominis preparations (Tamiya and Arai, 1966; Chang and Lee, 1963; Su et al., 1967). The response to microiontophoresis of acetylcholine or other cholinergic agonists in isolated frog sartorius muscle preparations is also blocked by α-BuTx as well as curare (Miledi et al., 1971; Lester, 1970). α-Bungarotoxin depresses endplate and miniature endplate potentials without affecting impulse conduction in the nerve endings or the passive electrical properties of the muscle membrane (Chang and Lee, 1966; Lester, 1970). All of these findings indicate that the neuromuscular block produced by α-BuTx is very similar to that of curare. They differ, however, in that the α-BuTx block is not abolished by anti-cholinesterase agents or prolonged washout periods of the blocked tissues.

Autoradiographic studies have demonstrated that α-BuTx, as well as cobra α-neurotoxins, labelled with ^{125}I accumulates almost exclu-

sively at the endplates of mouse and rat diaphragm muscle (Lee and Tsing, 1966; Lee et al., 1967; Fambrough et al., 1972) in the same manner as curare (Waser and Luthi, 1957). α-BuTx binding to the endplates of human muscles has been demonstrated using horseradish peroxidase-bound α-BuTx (Bender et al., 1976a, 1976b; Engel et al., 1977). Denervated rat hemidiaphragms bind α-BuTx over the entire muscle surface, while binding is restricted to the endplate region in the innervated control side (Lee et al., 1967). This is consistent with the physiologic spread of sensitivity to acetylcholine following denervation (Miledi, 1960).

The electric organs of fish such as Electrophorous electricus and Torpedo marmorata are composed of cells (electroplaques) which are derived embryologically from skeletal muscle (Keyes and Martins-Ferreira, 1953) and are richly innervated solely by cholinergic nerves on the ventral face (Feldberg and Fessard, 1942). Electrophysiologic and pharmacologic studies show these cells to possess acetylcholine receptors very similar to those found in skeletal muscle (Schonffeniels and Nachmansohn, 1957; Bennett, 1970). The acetylcholine receptor extracted from the electric organ of Electrophorous electricus and Torpedo marmorata binds labelled α-BuTx. Binding is inhibited by curare, acetylcholine and carbachol (Su et al., 1967; Changeux et al., 1970; Lester, 1970, 1971; Miledi et al., 1971). Similar findings have been reported for solublized AChR extracted from striated muscle (Miledi and Potter, 1971; Berg et al., 1972) and brain (Bosman, 1972; McQuarrie et al., 1978; Tindall et al., 1978).

Simultaneous autoradiography for AChR with ^{3}H-α-BuTx and acetyl-

cholinesterase with ^{3}H-DFP (diisopropylfluorphosphate) indicate that α-BuTx does not bind to acetylcholinesterase (Barnard et al., 1971). Furthermore, labelling of α-BuTx with ^{131}I, ^{125}I or ^{3}H does not appear to alter its toxicity or binding to AChR extracted from electric organ or skeletal muscle (Miledi et al., 1971; Berg et al., 1972; Eldefrawi and Fertuck, 1972; Vogel et al., 1972; Clark et al., 1972), although there is some question regarding the specificity of binding of radio-labelled α-BuTx to AChR extracts of brain and sympathetic ganglia (Enterovic et al., 1975; Lukasiewicz et al., 1978).

C. USE OF CONCANAVALIN-A AS A PROBE FOR THE NICOTINIC ACETYLCHOLINE RECEPTOR

Concanavalin-A (Con-A) is a non-carbohydrate containing protein extracted from the jack bean, Canavalia ensiformis (Sumner, 1919). Con-A is capable of agglutinating erythrocytes (Sumner and Howell, 1936a), and hence is classified as a lectin. In addition to agglutinating red blood cells, Con-A possesses many other biological properties, all of which result from the binding of Con-A to a specific carbohydrate moiety on the membrane of the cell(s) with which it interacts (Sharon and Lis, 1972). Con-A has been found to stimulate certain membrane-bound enzymes (Traficante et al., 1978; Riordan et al., 1977; Caraway et al., 1975), as well as stimulate the transformation of mouse lymphocytes from small "resting" cells into large blast-like cells which ultimately undergo mitotic division, i.e., Con-A is mitogenic (Schechter et al., 1976; Prujansky et al., 1978). In addition, Con-A binds to variant or transformed cell lines in a manner different from its binding to the parent cell lines (Imbar and Sachs, 1969a, 1969b; Ceri and Wright, 1971). These studies illustrate the use of Con-A to investigate the relationship of membrane-bound proteins with cellular function, as well as morphological differences between the membranes of native and transformed cells.

The requirements for the interaction of Con-A with polysaccharides and glycoproteins have been extensively studied by Goldstein and his associates (Goldstein et al., 1965a, 1965b; Goldstein and So, 1965; So and Goldstein, 1968; Smith and Goldstein, 1967; Poretz and Goldstein, 1970). Con-A binds very tightly to α-D-mannopyranose and α-D-glucopyranose, their glycosides and glycoproteins containing

these saccharides (Poretz and Goldstein, 1970). The carbohydrate combining site of Con-A appears directed primarily toward the hydroxyl groups at the C-3, C-4 and C-6 positions. Additionally, α-linked sugars appear to bind more strongly than the corresponding β forms.

Con-A, as well as most lectins, contains Mn^{2+} and Ca^{2+} which are necessary for carbohydrate binding (Sumner and Howell, 1936b; Yariv et al., 1968). Removal of these metal ions by exposure to acid pH, which does not denature Con-A, abolishes carbohydrate binding activity (Agrawal and Goldstein, 1968). Equilibrium sedimentation (Kalb and Lustig, 1968) and equilibrium dialysis studies (Yariv et al., 1968; Kalb and Levitski, 1968) indicate that the Con-A molecule consists of subunits (protomers) of approximately 30,000 daltons. Each subunit contains one carbohydrate binding site and is composed of 238 amino acids (Edelman et al., 1972). The state of aggregation of the Con-A complex is pH dependent. Con-A exists as a tetramer at pH values greater than 7, but as a dimer at pH values below 6 (Yariv et al., 1968; Kalb and Levitski, 1968). These findings have been verified by x-ray crystallographic studies (Edelman et al., 1972; Greer et al., 1970; Hardman et al., 1971, 1972; Quiocho et al., 1971; Reeke et al., 1972; Becker et al., 1971). At pH 6.8, four identical subunits interact to form a pseudotetrahedral complex. One saccharide is bound to each subunit, so that four sugar molecules occupy symmetrically equivalent positions on the Con-A tetramer (Edelman et al., 1972; Becker et al., 1971). Edelman et al. (1972) maintain that the distances between the saccharide binding sites on the tetramer are consistent with possible distances of closest ap-

proach of cells in the agglutination reaction and formation of precipitates with polysaccharides and glycoproteins by cross-linking of these compounds by Con-A.

Lens culinaris is a lectin isolated from lentil seeds. Although not as extensively studied as Con-A, Lens culinaris has been found to be mitogenic (Toyoshima et al., 1970; Young et al., 1971), has a subunit composition (Ticha et al., 1970; Howard et al., 1971) and contains Mn^{2+} and Ca^{2+} (Paulova et al., 1971; Tichy et al., 1971). Con-A and Lens culinaris also share the same carbohydrate specificity (Howard and Sage, 1969), however the affinity of Lens culinaris for α-D-mannose and α-D-glucose is less than that of Con-A (Stein et al., 1971), which offers advantages in isolation of glycoproteins using lectin affinity chromatography (Hayman and Crumpton, 1972; Hayman et al., 1973; Kinzel et al., 1976).

Lectins, in particular Con-A, have been used to study the glycoprotein nature of acetylcholine receptor extracted from Electrophorous electricus (Meunier et al., 1974), Torpedo californica (Mattson and Heilbronn, 1975; Vandlen et al., 1976; Raftery et al., 1976), rat brain (Salvaterra et al., 1977), and mammalian skeletal muscle (Brockes and Hall, 1975b; Almon and Appel, 1976). The carbohydrate composition of the acetylcholine receptor extracted from the electric organ of Torpedo californica was determined by gas chromatography, which also allowed quantitation of the various saccharides present. Carbohydrates were found to contribute 3.8 ± 1.8% of the weight of the receptor (Heilbronn and Mattson, 1974; Mattson and Heilbronn, 1975). Mannose, galactose, glucose and N-acetyl-glucosamine contributed 80%, 18%, 2% and 0.01%, respectively, of the

carbohydrate present. Raftery et al. (1976) and Vandlen et al. (1976) confirmed the presence of these saccharides using carbohydrate specific lectin binding to AChR also extracted from Torpedo californica. Receptor binding to the lectins Con-A, Lens culinaris and Ricinus communis was used by Meunier et al. (1974) to demonstrate the presence of mannose, glucose and N-acetyl-glucosamine in a highly purified receptor preparation extracted from the electric organ of Electrophorous electricus.

Con-A has been used to demonstrate the glycoprotein nature of the AChR extracted from mammalian skeletal muscle (Brockes and Hall, 1975b; Almon and Appel, 1976). Assay procedures using the binding of the receptor to Con-A have also been developed (Mittag et al., 1976; Hall and Reiness, 1975) which are comparable to and much more convenient than the standard AChR assay procedures (Mittag et al., 1976).

Lectin affinity chromatography using Con-A, as well as Lens culinaris, immobilized on Sepharose gels has been used to purify AChR extracted from mammalian skeletal muscle (Almon and Appel, 1976; Froehner et al., 1977a; Shorr et al., 1978) and the electric organ of Torpedo californica (Mattson and Heilbronn, 1975). Elution of 40% to 60% of the receptors applied to these lectin affinity columns with α-methyl-D-mannoside (Almon and Appel, 1976; Froehner et al., 1977a; Mattson and Heilbronn, 1975) strongly suggests heterogeneity of the receptor population based on differences in the carbohydrate content and orientation in the receptor. This point is also demonstrated by the ability of soluble Con-A to inhibit 60% of the binding of a cobra α-neurotoxin to AChR extracted from the electric

organ of Electrophorous electricus (Meunier et al., 1974). These studies suggest that Con-A is a useful tool which can be used to study the molecular biology of the AChR.

D. NICOTINIC ACETYLCHOLINE RECEPTOR

In the fluid mosaic model of membranes, advanced by Singer (Singer, 1971, 1974; Singer and Nicolson, 1972), the fluid phospholipid is regarded as a matrix into which is incorporated the proteins which make up the major constituents of functional membrane (Guidotti, 1972). The membrane proteins (including their carbohydrate components) are categorized as integral or peripheral and are distinguished by several criteria (Singer, 1974). Peripheral proteins can be dissociated from the membrane with mild treatment (e.g. high ionic strength or chelating agents), are usually lipid free when isolated and are soluble in aqueous media. Integral proteins require vigorous treatment for dissociation from membranes (e.g. detergents), are usually associated with lipid when isolated and are usually insoluble or aggregate in aqueous media. Using these criteria, the acetylcholine receptor, as well as most neurotransmitter and hormone receptors studied to date, would be classified as integral proteins (Singer, 1974).

The Singer-Nicolson model of the fluid membrane allows for the mobility of membrane constituents both in the plane of the membrane (lateral diffusion) and perpendicular to the plane of the membrane, (Singer, 1974). This concept of membrane fluidity is heavily supported by studies measuring lateral diffusion rates of surface antigen receptors (Frye and Edidin, 1970; Nicolson et al., 1971; Edidin and Fambrough, 1973) and lectin receptors in lymphocytes (Edelman et al., 1973; Nicolson, 1973; Rosenblith et al., 1973). Lateral movement of acetylcholine receptors has recently been demonstrated in single embryonic frog muscle cells (Orida and Poo, 1978).

The mobility of AChR in developing muscle cell membranes is thought to play an important role in synaptogenesis. During muscle cell development, mononucleate myoblasts differentiate and fuse to form multinucleate myotubes. The myoblasts are not sensitive to acetylcholine nor do they bind α-bungarotoxin (Kano et al., 1971; Kano and Shimada, 1972). Once they differentiate into myotubes however, chemosensitivity to acetylcholine and α-bungarotoxin binding appear and are uniformly distributed along the entire membrane (Diamond and Miledi, 1962; Fishbach and Cohen, 1973a; Hartzell and Fambrough, 1972; Vogel et al., 1972; Steinbach et al., 1973). Receptor clustering on the developing muscle membrane is thought to initiate synapse formation. Mapping of AChR during development of cultured chick muscle cells in absence of neurons shows clustering of receptors with a very high density (approximately 9000 α-BuTx sites/μm^2) compared to background of approximately 200 sites/μm^2 (Vogel et al., 1972; Sytkowski et al., 1973). Receptor localization using electrophysiological techniques reveals similar findings (Fishbach and Cohen, 1973a, 1973b; Fishbach et al., 1974a, 1974b). When chick muscle cells are cultured in the presence of spinal cord cells, they form functional cholinergic synapses in the areas of high receptor localization (Fishbach, 1972; Fishbach and Cohen, 1973a, 1973b; Fishbach et al., 1974a, 1974b). Once the synapse is formed, the area of acetylcholine sensitivity and α-bungarotoxin binding to receptors is restricted almost exclusively to the neuromuscular junction.

In normal muscle, the nerve exerts some influence to prevent the appearance of extrajunctional receptor (Guth, 1968; Harris, 1974; Mathers and Thesleff, 1978). Within several days after denervating a

muscle, extrajunctional AChR appear (Axelsson and Thesleff, 1959; Miledi and Potter, 1971; Barnard et al., 1971; Berg et al., 1972; Fambrough and Hartzell, 1972). This great increase in density and distribution of extrajunctional AChR is the basis of the classical phenomenon of denervation supersensitivity (Cannon and Rosenblueth, 1949). If reinnervation of the muscle occurs, a reduction in the number of extrajunctional receptors and area of acetylcholine sensitivity occurs until the muscle returns to its normal innervated state (Miledi, 1960b; McArdle and Albuqurque, 1973). The appearance of denervated extrajunctional receptors does not represent a simple lateral redistribution of junctional receptors. There is no decrease in endplate sensitivity to acetylcholine during the development of supersensitivity (Miledi, 1960a; Hartzell and Fambrough, 1972; Dryer and Peper, 1974). The actual determination of the number of receptors in the innervated and denervated states shows a 20 to 30 fold net increase in the number of receptors in denervated muscle (Miledi and Potter, 1971; Hartzell and Fambrough, 1972; Berg et al., 1972). Furthermore, it has been demonstrated by Devrotes and Fambrough (1976), and Brockes and Hall, (1975c), using incorporation of labelled amino acids, that the appearance of extrajunctional receptors in cultured denervated rat diaphragm results from a de novo synthesis of receptors. No incorporation of labelled amino acids was detectable in innervated diaphragms. This is consistent with earlier work which showed that inhibition of protein and RNA synthesis prevents the appearance of extrajunctional AChR following denervation (Fambrough, 1970; Grampp et al., 1972).

The similarities between the events of synaptogenesis of cul-

tured embyronic muscle and reinnervation of denervated muscle have stimulated much interest in the process of synapse formation and the nature of denervated junctional and extrajunctional AChR and their relationship to neonatal receptors (see review by Brockes et al., 1976).

Comparison of the physiological and pharmacological properties of AChR's found in innervated and denervated muscle reveal that junctional and extrajunctional receptors are very similar. Whole muscle studies show that both mediate increases in K^+ and Na^+ permeabilities and exhibit pharmacological properties of classical nicotinic receptors (Axelsson and Thesleff, 1959; Jenkinson and Nicholls, 1961; Beraneck and Vyskoscil, 1967). However, there are significant differences between the two. A number of workers have reported that extrajunctional receptors are less sensitive to curare than junctional receptors (Beranek and Vyskoscil, 1967; Chiu et al., 1974; Lapa et al., 1974). Katz and Miledi (1972), and Peper and Dreyer (1976) have used AChR noise analysis to demonstrate differences between junctional and extrajunctional receptor in the length of time ion channels remain open during the elementary conductance change. In addition, Berg and Hall (1974, 1975) have shown that extrajunctional receptors have a much faster turnover rate than junctional receptors.

Brockes and Hall (1975a, 1975b) have compared solublized AChR extracted from innervated and denervated rat diaphragm muscle. No differences in the size or shape of the receptors could be found using zonal centrifugation on sucrose density gradients or agarose gel filtration. Both junctional and extrajunctional receptors interact extensively with Concanavalin-A, indicating that both are glyco-

proteins containing α-D-mannoside and/or α-D-glucoside residues. When rabbit serum raised against purified receptor from Electrophorous electricus was incubated with each of the two preparations of toxin-labelled receptors, the precipitation curves obtained were identical, indicating that the antigenic determinants that react with this serum are common to both junctional and extrajunctional receptors. However, incubation of receptors extracted from rat skeletal muscle with sera from patients with myasthenia gravis (Almon and Appel, 1975; T.W. Mittag and J. Tabachnick, unpublished) clearly distinguishes junctional from extrajunctional receptors. Myasthenic sera have a much higher titer against extrajunctional receptor as compared to juncitonal receptor.

Although 90% inhibition of α-BuTx binding to both junctional and extrajunctional receptors could be achieved by curare, the apparent K_D's were found to be different (5.5×10^{-7} M for extrajunctional receptor and 4.5×10^{-8} M for junctional receptor). This confirmed the physicological experiments mentioned earlier citing a decrease in curare sensitivity of denervated muscles.

Finally, isoelectric focusing on polyacrylamide gels reveals that the junctional receptor has an isoelectric point about 0.15 pH unit lower than the extrajunctional receptor (5.27 vs 5.12). Based on their findings, Brockes and Hall concluded that although junctional and extrajunctional receptors are very similar, they are structurally distinct molecules.

A comparison of receptors extracted from the diaphragms of adult and neonatal rats indicates that receptors present in neonatal muscle are identical with those found in denervated adult muscle

(Brockes and Hall, 1975b). These investigators found that neonatal extrajunctional receptor exhibited the same sensitivity to curare as adult extrajunctional receptor (K_{Dapp} for neonatal AChR = 4.1×10^{-7} M vs 5.5×10^{-7} M for adult extrajunctional receptor). Isoelectric focusing of neonatal junctional and extrajunctional receptor gives a bimodal distribution very similar to that of adult denervated muscle corresponding to junctional and extrajunctional receptors.

Brockes and Hall suggest that the two forms of receptor (junctional vs extrajunctional) are the product of different genes, or alternatively, that extrajunctional receptors may be precursors of junctional receptors in developing muscle fibers and in adult denervated fibers. They note that Langley (1905) in his original paper on "receptive substance" considered the possibility that extrajunctional receptors were necessary for synapse formation and were chemically modified by neuronal influence into the junctional form of the receptor. The role of neuronal interaction and extrajunctional acetylcholine receptors in synaptogenesis awaits further clarification.

An interesting aspect of the work of Brockes and Hall (1975a) was that the kinetics of binding of α-BuTx to the receptor and dissociation of toxin-receptor complexes could not be explained by assuming a homogeneous population of receptors when examining extrajunctional or junctional receptors. In each case, it appeared that two kinetically distinct populations of toxin binding sites were present despite the fact that during isoelectric focusing both migrated as single bands. Brockes and Hall suggested that such findings could result from a) two populations of receptors distin-

guishable by kinetics of toxin binding or b) a homogeneous population of receptors, each containing two different toxin binding sites. This second possibility is quite feasible in the light of work done with AChR extracted from electric fish suggesting two toxin binding sites per AChR molecule (see reviews by Karlin, 1974, 1977; Rang 1975; Cohen and Changeux, 1975; Eldefrawi and Eledefrawi, 1977).

Although there are many studies on the detailed subunit structure of nicotinic AChR extracted from Electrophorous and Torpedo (see reviews by Karlin, 1975, 1977; Cohen and Changeux, 1975; Rang, 1975; Eldefrawi and Eldefrawi, 1977), such work on mammalian receptors has been hampered by the low yield of receptor in preparations extracted from innervated and denervated muscle relative to those obtained using electric organ. In mammals, extrajunctional receptors, because of their greater numbers in denervated muscle, are easier to extract and purify than junctional mammalian muscle receptors. Isolation techniques utilizing lectin affinity chromatography may offer a solution to this problem (Shorr et al., 1978; Froehner et al., 1977a).

A number of investigators have determined that the receptor found in Electrophorous electricus and Torpedo manorata is a multisubunit complex consisting of at least 4 different peptides of 40,000 to 110,000 daltons (review by Karlin, 1977). Only the 40,000 dalton subunit has a known function, that of binding α-BuTx and presumably this subunit corresponds to the acetylcholine binding site (Karlin, 1977). Witzemann and Raftery (1978) have demonstrated conformational changes in the higher molecular weight subunits when α-BuTx or a cholinergic ligand binds to the 40,000 dalton subunit of AChR extract-

ed from Torpedo mamorata. The implication of these conformational changes in the higher molecular weight subunits and their function remains unclear at present, but it does indicate that these higher molecular weight subunits belong to a supramolecular complex of interacting polypeptides associated with the postsynaptic membrane and are not artifacts due to incomplete proteolysis of the 40,000 dalton subunit.

Studies of the subunit structure of AChR extracted from denervated rat hindlimb muscle using SDS-polyacrylamide gel electrophoresis reveal two major components of 45,000 and 51,000 daltons, both of which bind α-BuTx (Froehner et al., 1977b), along with minor components of 49,000, 56,000, 62,000 and 110,000 (Froehner et al., 1977a). These findings indicate that mammalian nicotinic receptor may be more complex than AChR extracted from Torpedo or Electrophorous. However, similar studies of AChR extracted from denervated rat hindlimb muscle (Shorr et al., 1978) and embryonic calf skeletal muscle differentiated in tissue culture (Merlie et al., 1978), show only one subunit of 41,000 daltons. Merlie et al. (1978) claim that there are 3 isoelectric forms of this subunit although the nature of this heterogeniety is unknown at present. Shorr et al. (1978) also found only the 40,000 dalton subunit in their analysis of Torpedo receptor, in disagreement with many other workers (see Karlin, 1977). Whether these discrepancies result from species differences, differences in analytical techniques, or proteolytic problems during purification remains to be determined.

E. MYASTHENIA GRAVIS

Myasthenia Gravis (MG) is a neuromuscular disorder characterized by excessive weakness and fatigability of skeletal muscles which is temporarily relieved by rest and anticholinesterase agents. This latter finding and the similarities between MG and curare poisoning suggested that the site of the disorder was the neuromuscular junction (Walker, 1934). This idea was later supported by the electrophysiological studies of Elmqvist et al. (1964), who showed that the miniature endplate potentials (mepps) at the neuromuscular junctions of patients with MG were of smaller amplitude than those of normal patients. However, the response of these muscles to bath applied carbamlycholine was normal. This suggested that the number of acetylcholine (ACh) molecules in each vessicle was decreased, and that the site of the disorder was presynaptic. However, as reviewed by several authors (Grob, 1976; Elias and Appel, 1976; Drachman, 1978; Drachman et al., 1977), no other direct evidence exists for a presynaptic defect, as ACh synthesis, packaging and release all appear normal in myasthenic patients. Furthermore, as noted by Drachman et al. (1976), reduced mepp amplitude can be caused by a decrease in the concentration of functional AChR, resulting from either a decrease in available ACh or AChR or both.

The hypothesis suggesting a presynaptic defect was made less tenible by experiments which provided direct measurement of the number of AChR on muscle endplates in normal and myasthenic muscle. Such work was made possible by the use of radiolabelled α-neurotoxins. Using ^{125}I-α-BuTx, Fambrough et al. (1973) demonstrated a significant reduction in the number of AChR per neuromuscular junction in the

muscles of myasthenics. Morphological studies also indicate a postsynaptic defect in the pathophysiology of MG. Ultrastructural examination shows a decreased endplate surface area and a loss of postjunctional folds in the muscles biopsied from patients with MG (Wolf, 1966; Engel and Santa, 1971; Engel et al., 1977). Bender et al. (1976a, 1976b) and Engel et al. (1977) have demonstrated decreased or absence of horseradish peroxidase-bound α-BuTx to muscles biopsied from myasthenic patients, indicating a block of, or decrease in, the number of receptors at the neuromuscular junction.

Further evidence for a postjunctional defect is provided in an animal model of MG produced by injecting rats with α-cobra toxin. These animals all showed the characteristic signs and symptoms of MG (Satyamurti et al., 1975). Since it is known that α-cobra toxin binds to AChR and causes a decrease in the number of functional receptors, this model strongly suggests that the characteristic signs of human MG can be caused by a postsynaptic defect affecting the number of functional receptors.

The animal model discovered by Patrick and Lindstrom (1973) not only strengthened the postsynaptic hypothesis, but also shed light on the possible cause of the defect. They showed that rabbits injected with highly purified AChR from Electrophorous electricus developed characteristics similar to human MG, and produced antibodies to the purified eel AChR. These antibodies were found to cross-react with rabbit receptors. The same result has been produced in goats, monkeys, rats and mice (Lennon, 1976). These results indicate that MG can result from a postsynaptic defect, and that it may be an autoimmune disease, as suggested by Simpson (1960). He proposed an auto-

immune basis for MG based on the high incidence of thymic hyperplasia and thymoma in myasthenics, as well as the improvement seen in MG patients undergoing thymectomies and/or immunosupressive therapy.

The autoimmune basis for MG has gained much support in the past few years due to the discovery of anti-receptor antibodies in the serum of the majority of patients with MG (Almon et al., 1974a; Appel et al., 1975; Bender et al., 1975; Lindstrom et al., 1976a; Mittag et al., 1976). The origin of these anti-receptor antibodies and the nature of their interaction with receptors in the pathogenesis of MG remains unclear. This is due in part to the different sources of AChR and anti-receptor antibody used by various investigators (Lennon, 1976). As demonstrated by Lindstrom and his colleagues (Lindstrom, 1976; Lennon et al., 1975, 1976; Lindstrom et al., 1976b, 1978), although the antibodies to AChR show partial species cross-reactivity, the extent of serologically detectable cross-reactivity between receptors of different species appears to be small. For example, Brockes and Hall (1975b), as mentioned earlier, reported that rabbit antiserum to eel receptor formed immune complexes with junctional and extrajunctional rat muscle AChR equally well. This implies that junctional and extrajunctional receptors are antigenically similar. However, human myasthenic anti-receptor antibodies are able to distinguish between junctional and extrajunctional receptors (Almon and Appel, 1975, 1976b; Mittag and Tabachnick, unpublished). Human myasthenic antibodies have been shown to cross-react fairly well with AChR extracted from monkey and fetal calf muscle (Lindstrom et al., 1978), as well as denervated rat muscle (Almon and Appel, 1975, 1976b; Mittag and Tabachnick, unpublished).

However, very little cross-reactivity with eel, Torpedo or innervated rat muscle receptors is observed. Therefore, when using human myasthenic sera as a source of anti-receptor antibodies, one should use AChR from a species of animal that will cross-react well with human sera if human muscle is not available. Such considerations are important in trying to determine the nature of the antibody-receptor interaction in the pathogenesis of MG.

Several types of interaction of human myasthenic anti-receptor antibody with AChR derived from several sources have been described. Almon et al. (1974) found that the sera of 5 of 15 myasthenics inhibited α-BuTx binding to solubilized receptor extracted from denervated rat muscle by up to 50% (Almon and Appel, 1975). Mittag et al. (1976) found toxin binding inhibitory activity in only 2 of 28 patients, using the same receptor source. No inhibition of toxin binding was observed for receptors extracted from normal human (Lindstrom et al., 1976d), or innervated rat muscle (Almon and Appel, 1975, 1976b), or Torpedo electric organ (Aarli et al., 1976). Bender et al. (1975, 1976a) demonstrated a toxin blocking factor in 75% of myasthenic sera using whole normal human and denervated rat muscles incubated with myasthenic sera or IgG. Lennon (1976) has suggested that human or rat extrajunctional AChR (preferrably in their membrane environment) appear to be the required antigen for demonstrating the toxin blocking factor in myasthenic sera. Furthermore, demonstration of this factor using solubilized receptor may depend on the conformation assumed by the receptor in solution and would therefore be dependent on what type and concentration of detergent is used to solubilize the receptor. These differences in

receptor preparation and animal source may explain why blocking factor is observed by some workers but not others.

Immunoprecipitation of solubilized AChR labelled with ^{125}I-α-BuTx is another property ascribed to myasthenic sera. Anti-receptor antibodies have been detected in over 85% of patients with MG using this method (Lindstrom et al., 1976a; Almon and Appel, 1976b; Mittag et al., 1976). Since the receptors are labelled with toxin prior to exposure to myasthenic sera, these antibodies are directed to antigenic determinants other than the toxin binding site and may represent a class of antibody different than that which inhibits the binding of toxin to AChR. In all three studies mentioned, 100% of the receptors can be immunoprecipitated by this form of myasthenic antibody, indicating that the antibody recognizes an antigen common to all AChR.

Mittag et al. (1976) have reported that 65% of the myasthenic sera screened in their study inhibited the binding of solubilized rat extrajunctional receptors to Con-A-Sepharose affinity columns, indicating that a class of myasthenic antibody(s) interacts with carbohydrate moieties (α-D-mannoside or α-D-glucoside) of the receptor. A 50% maximal inhibition of Con-A-Sepharose binding was observed. Furthermore, when tested in parallel with the immunoprecipitation method of detecting anti-receptor antibodies, there was a significant lack of concordance between the two methods in certain patients, suggesting that more than one antibody type interacts with the receptor.

Finally, myasthenic antibodies (sera or IgG) have been found to significantly increase the rate of receptor degradation both <u>in vitro</u>

(Appel et al., 1977; Heinemann et al., 1977; Kao and Drachman, 1977a) and in vivo (Stanley and Drachman, 1978). This property has been attributed to the ability of the antibodies to cross-link acetylcholine receptors and somehow specially select these receptors for degradation (Drachman et al., 1978a).

The animal models of myasthenia gravis (Lindstrom et al., 1976; Seybold et al., 1976; Lennon et al., 1976) suggest that the antibodies trigger a complement mediated autoimmune attack of the receptor, (Lennon et al., 1978) resulting in the altered morphology described previously. However, the exact role of the antibodies in the pathogenesis of human MG remains to be ascertained. A more intriguing question concerns the origin of the autoimmune response. The most popular hypothesis points to the involvement of the thymus, however its exact role is not understood. The pathologic changes found in the thymuses of myasthenic patients (Castleman, 1966) as well as the beneficial effects of thymectomy (Papatestas et al., 1971) first suggested thymic involvement in the pathogenesis of MG. Approximately 75% of all myasthenics exhibit some type of thymic pathology. 85% of these patients show hyperplastic changes in the form of thymic germinal centers, while the remaining 15% have overt thymomas (Castleman, 1966; Papastestas et al., 1971; Namba et al., 1976). Thymus glands from rats have been found to contain myoid cells which develop into typical muscle cells when cultured (Van de Velde and Freidman, 1970; Werkerle et al., 1975). These cells exhibit α-BuTx binding and are sensitive to acetylcholine indicating the presence of AChR (Kao and Drachman, 1977b). Aharonov et al. (1975) and Lindstrom et al. (1976d) have found AChR in thymic extracts. It has been suggested that a

break in tolerance might result from alteration of either thymic lymphocytes and/or thymic myoid cells, making the AChR antigenic and initiating an autoimmune attack on the AChR at the neuromuscular junction (Simpson, 1960, 1978; Aharonov et al., 1975; Kao and Drachman, 1977b). Presumably thymectomy somehow interferes with this process and thus results in an improvement in the condition and prognosis of many myasthenics (Papetestas et al., 1971). However, Namba et al. (1978) have reported 33 cases of MG developing two weeks to six years (mean of 1.5 years) after removal of thymoma and adjacent thymus. These authors also note that the life span of thymic lymphocytes is probably several years and the peripheral pool of these lymphocytes may be the link in an indirect causal relationship between the thymus and development of MG. The event that initiates the activation of this peripheral pool of lymphocytes and the subsequent autoimmune response is unknown.

II. SPECIFIC AIMS

There are several suggestions in the literature reviewed to support the concept of receptor heterogeneity in solubilized receptor preparations extracted from denervated rat muscle.

a) Brockes and Hall (1975a) have demonstrated the existence of two populations of kinetically distinguishable toxin binding sites in denervated rat diaphragm. Each population is present in roughly equal amounts.

b) Almon and Appel (1976) and Froehner et al. (1977) reported partial elution of AChR from lectin affinity columns, while Mittag et al. (1976) demonstrated a 50% maximal inhibition of receptor binding to Con-A-Sepharose following exposure to myasthenic sera. These studies suggest two populations of receptor, present in equal amounts and distinguished by differences in their carbohydrate moieties.

c) Almon et al. (1974a, 1975) reported a 50% maximal inhibition of toxin binding to receptors exposed to myasthenic sera prior to toxin labelling. This also suggests two populations of equal size differing in susceptibility to toxin block by myasthenic antibodies.

In addition, heterogeneity of the anti-receptor antibodies found in myasthenic sera has been observed. Mittag et al. (1976) demonstrated the presence of three types of anti-receptor antibodies in myasthenic sera which 1) inhibit α-BuTx binding to receptors, 2) inhibit the binding of labelled receptors to Con-A-Sepharose, and 3) form complexes with labelled receptors which are precipitated with anti-human IgG. (In this study, these antibodies will be refer-

red to as factor-B (MG-B), factor-S (MG-S), and factor-I (MG-I), respectively.)

The objective of this study is to confirm and explore the nature of this suggested receptor and antibody heterogeneity by examining the interactions of soluble and immobilized lectins, and myasthenic antibodies with AChR extracted from denervated rat hindlimb muscle. Using lectin affinity chromatography, physical separation of two forms of toxin binding sites, differing in kinetics of toxin binding and affinity to immobilized lectins, will be shown. Immunological techniques will be utilized to demonstrate a) the existence of at least three types of anti-receptor antibody in the sera of myasthenic patients and b) the existence of subclasses of AChR in the receptor populations distinguished by lectin, antibody and toxin binding techniques. A model of the receptor based on these differences will be proposed.

III. METHODS

A. PREPARATIONS

1. Acetylcholine receptor preparation using hydroxylapatite chromatography

Six female Sprague Dawley rats (225-249 gms) were sacrificed 10 to 14 days following bilateral sectioning and removal of a one centimeter segment of the sciatic nerve. The denervated hindlimb muscles were removed immediately and minced in a solution containing 10 volumes 1 M KBr and 1 mM methane sulfonyl fluoride (MSF) per gram wet weight, homogenized with a Brinkman Polytron homogenizer (setting 2 for 1 minute), passed through a 1 mm nylon net and centrifuged at 5000 xg for 10 minutes. The pellet was resuspended in a solution containing 5 volumes 1 M KBr and 1 mM MSF per gram wet weight, and centrifuged at 5000 xg for 10 minutes. The final pellet was extracted overnight at $4^{\circ}C$ with 2 volumes 2% (wt/v) Triton X-100 and 1 mM MSF. Following centrifugation at 39,000 xg for 30 minutes, the supernate was filtered through a Whatman GF/B glass-fiber filter and applied to a 50 ml spheroidal hydroxylapapite chromatography column which had been equilibrated with Buffer A (100 mM NaCl, 10 mM Tris-HCl (pH = 7.4), 1 mM EDTA, 1% (wt/v) Triton, 0.02% (wt/v) NaN_3). The column was thoroughly washed with Buffer A and the receptors were eluted with Buffer B (150 mM Na/PO_4 buffer (pH = 7.4), 1% Triton X-100, 1 mM EDTA, 0.02% NaN_3). The 2 fractions (15 to 18 ml/fraction) containing most of the receptors (as determined by assay) were concentrated using an Amicon Ultrafiltration Concentration Cell with an XM-100 A filter (MW cutoff = 100,000) under 25 to 50 psi nitrogen to a final volume of 0.5 ml per gram original wet weight of the

denervated muscles.

2. Purification of AChR using Lens culinaris-Agrose affinity chromatography

Receptors prepared using spheroidal hydroxylapatite chromatography were further purified by affinity chromatography on Agrose-bound Lens culinaris (P-L Biochemicals). Approximately 14 picomoles of AChR were recirculated 10 times through a 1 ml column of Lens culinaris-Agrose (LCA), which previously had been equilibrated with 150 mM Na/PO_4 buffer (pH = 7.4), 1% Triton X-100, 1 mM EDTA, 0.02% NaN_3. The column was washed with 2 ml of a solution containing 50 mM mannose. Two ml of 50 mM NaCl, 1% Triton X-100, 500 mM mannose were recirculated through the column system for 12 hours at 4°C using a peristalic pump and the columns were then separated. The hydroxylapatite column was washed with 2 ml fractions of 100 mM NaCl, 10 mM Tris-HCl (pH = 7.4), 1% Triton X-100 1 mM EDTA, 0.02% NaN_3 until the protein content of the fractions (determined spectrophotometrically, λ = 280) was negligible. The receptors were eluted with 150 mM Na/PO_4 buffer (pH = 7.4), 1% Triton X-100, 1 mM EDTA, 0.02% NaN_3 in fractions of 2 ml until the protein content of the fractions was negligible.

All fractions and washes were assayed for AChR content using the HTP column method, which will be described later in this section. In addition, the Lens culinaris column was labelled with ^{125}I-α-BuTx for 24 hours at 4°C to determine how much receptor remained bound to the column. The amount of toxin used was four times the total amount of AChR applied to the Lens column, or 80 picomoles, and was in a volume of 300 µl (one void volume of the column). After label-

ling, the column was washed with 20 ml 5 mM Na/PO_4 buffer (pH = 7.4), 150 mM NaCl, 1% Triton X-100 to removed free toxin and counted in the Beckman Biogamma counter.

3. Purification of α-Bungarotoxin

α-Bungarotoxin (α-BuTx) was obtained from Miami Serpentarium. Aliquots of the freeze dried toxin (2 to 3 mg) were suspended in 1.5 ml distilled water and applied to a 6 ml SP-C25 Sephadex column for final purification, as described by Mittag et al. (1976). The column was washed with 25 mM Na/PO_4 buffer (pH = 7.4) and the toxin was eluted with 150 mM NaCl. The concentration of the resulting BuTx solution was determined spectrophotometrically (λ = 273 nm, ε = 8300). The purified toxin was stored at -20°C.

4. Iodination of α-Bungarotoxin

α-Bungarotoxin was iodinated using the method of David (1972). Fifty µl of a 50% suspension of Sepharose bound lactoperoxidase (Worthington Biochemicals) were thoroughly washed with 200 mM Na/PO_4 buffer (pH = 6.5) in 150 mM NaCl. The suspension was added to 5 mCi carrier-free Na ^{125}I (New England Nuclear). Following the addition of 12 nmoles α-BuTx, 12 nmoles H_2O_2 were added to the reaction gradually over a 90 minute period, during which the reaction vial was gently agitated. The contents of the reaction vial were quantitatively transferred with a total of 2 ml distilled water to a cascade system consisting of a 1 ml QAE-A25 Sephadex column over a 6 ml SP-C25 Sephadex column. QAE-A25 (chloride form) is a strongly basic anion exchanger, was used to remove unreacted $^{125}I^-$ from the reaction mixture. Since α-BuTx is a positively charged basic protein, it passes through the QAE-A25 column and binds to the SP-C25 (sodium

form). SP-C25 is a strongly acidic cation exchanger. The remaining non-α-BuTx constituents of the reaction mixture were removed by washing the SP-C25 column with 25 mM Na/PO_4 buffer (pH = 7.2). Iodinated α-BuTx was eluted with 150 mM NaCl. The 2 or 3 fractions (0.8 ml/fraction) containing the highest amount of labelled toxin (determined by counting a 5 µl sample from each fraction) were pooled and stored at 4°C. Specific activity (determined by isotope dilution) was 100 to 250 cpm/fmole α-BuTx.

5. Serum preparation

Freshly drawn blood specimens from type I and type II myasthenic patients were allowed to clot at room temperature and centrifuged. Serum was removed using a Pasteur pipette, was filtered through a Millipore 0.45 µ cellulose acetate filter, and stored at 4°C in 0.02% NaN_3.

Normal serum was prepared in a similar manner.

6. Purification of immunoglobulin-G using DEAE-Sephadex chromatography

Immunoglobulin G (IgG) from normal and myasthenic serum was purified using the method of Capra et al. (1973). Serum (usually 2 ml) was diluted with an equal volume of 50 mM Na/PO_4 buffer (pH = 7.85) and applied to a 12 ml DEAE-A25 Sephadex chromatography column. The IgG fraction was eluted with 12 ml 50 mM Na/PO_4 buffer (pH = 7.85) and concentrated in an Amicon B15 Macrosolute Concentrator (MW cutoff = 15,000) to 2 ml, filtered through a 0.45 µ Millipore cellulose acetate filter and stored at 4°C in 0.02% NaN_3. The concentration of the resulting IgG solution was determined spectrophoto-

metrically (λ = 280 nm, ε = 13.8 for a 1% IgG solution).

7. Purification of Immunoglobulin-G using Protein-A-Sepharose affinity chromatography

Immunoglobulin-G from serum and DEAE purified IgG fractions was purified using Protein-A-Sepharose (Pharmacia) affinity chromatography in a batch procedure. Two ml of serum or DEAE prepared IgG were recycled through 0.5 ml columns of Protein-A-Sepharose which had previously been equilibrated with 50 mM Na/PO_4 buffer (pH = 7.85). The columns were washed with 1.2 ml fractions of this buffer until the protein content of the fractions (determined spectrophotometrically) was negligible. The IgG adsorbed to the Protein-A column was desorbed with 1.2 ml fractions of 100 mM acetate buffer (pH = 3.0). Acetate fractions were collected until the protein content of the fractions was negligible and adjusted to pH = 7.0 with 1 M sodium-bicarbonate and dialyzed for 24 hours against 50 mM Na/PO_4 buffer (pH = 7.5). As a control, a solution of acetate buffered to pH = 7.0 with bicarbonate was also dialyzed. All fractions, as well as the filtrate recycled through the Protein-A columns and the native serum or IgG fractions were assayed using the Con-A/HTP cascade.

B. ASSAY PROCEDURES

Triton-solubilized toxin-receptor complexes have been shown to form soluble complexes with Con-A (Meunier et al., 1974; Brockes and Hall, 1975b). Mittag et al. (1976) found that toxin-receptor complexes from denervated rat muscle are strongly adsorbed to gels of immobilized Con-A on Sepharose, whereas free ^{125}I-α-BuTx does not adsorb to Con-A-Sepharose. Free α-BuTx can be separated from toxin-receptor complexes by washing the gel with 150 mM NaCl and 1% Triton X-100. The radioactivity remaining on the gel after washing with NaCl indicates how much toxin-receptor complex is present in a particular sample.

Klett et al. (1973) developed a receptor purification method consisting of α-cobra toxin affinity chromatography followed by chromatography on hydroxylapatite. As previously noted, the hydroxylapatite portion of this method has been used in this study to prepare AChR. However, as mentioned in the following Results and Discussion sections, toxin-labelled receptors cannot be eluted from hydroxylapatite columns. Free ^{125}I-α-BuTx does not bind to hydroxylapatite and can be separated from toxin-receptor complexes by washing the column with 200 mM Tris-HCl (pH = 6.0) and 1% Triton X-100 followed by 150 mM NaCl, 5 mM Na/PO_4 buffer (pH = 7.4) and 1% Triton X-100. Therefore, as was the case with the Con-A-Sepharose assay, the radioactivity remaining on the hydroxylapatite column indicates how much toxin-receptor complex is present in a given sample.

The following assay methods based on the procedures described above have been used in this study.

1. Concanavalin-A-Sepharose affinity column method

Approximately 50 µl from the various reaction mixtures (to be described) was applied to a 1 ml Concanavalin-A-Sepharose (Con-A) column. The column was washed with 10 ml 150 mM NaCl, 5 mM $PO_4^{\equiv}$ (pH = 7.4), 1% Triton X-100 (PBS/1%). The radioactivity remaining on the column was counted in a Beckman Biogamma Counter.

2. Hydroxylapatite adsorption column method

Approximately 50 µl from the various reaction mixtures was applied to a 1 ml column of hydroxylapatite (HTP). The column was washed with 5 ml PBS/1% and 5 ml Tris/1% (200 mM Tris-HCl (pH = 6.0), 1% Triton X-100).

3. Concanavalin-A/Hydroxylapatite cascade

Approximately 50 µl from the various reaction mixtures was applied to a 1 ml column of Concanavalin-A-Sepharose over a 1 ml hydroxylapatite column (Con-A/HTP cascade). The column cascade was washed with 5 ml PBS/1% and the columns were separated. The Con-A column was washed again with 5 ml PBS/1% and the HTP column was washed with 5 ml Tris/1%. The radioactivity remaining on the columns was counted in a Beckman Biogamma Counter.

The following immunological techniques were used to determine the amounts of Con-A-receptor complex and human IgG-receptor complex formed in several experiments.

4. Immunoprecipitation for Concanavalin-A

Samples from the various reaction mixtures were incubated 24 hours at 4°C with a volume of goat anti-Con-A antiserum (Miles Labs) containing at least a 10 fold molar excess of antibody relative to Con-A. Rabbit anti-goat IgG (Miles Labs), containing at least a 2.5 fold molar excess of antibody relative to goat antiserum, was added

to effect immunoprecipitation. Following another 24 hours at 4°C, the precipitate was washed 4 times with PBS/1% and counted in the Beckman Biogamma Counter.

5. Immunoprecipitation for human IgG

Samples from the various reaction mixtures were incubated for 24 hours at 4°C with a volume of goat anti-human gamma chain IgG (Cappel Labs) containing at least a 3 fold molar excess of anti-human IgG relative to human IgG. The precipitates were washed with 4 ml PBS/1% and counted in the Beckman Biogamma Counter.

C. EXPERIMENTAL

1. The binding of toxin-labelled receptors to Con-A, HTP and Con-A/HTP cascade columns: effects of incubation with Con-A

Duplicate samples of AChR (final concentration 3.1 nM) were labelled with ^{125}I-α-BuTx (final concentration 20 nM) for 24 hours at 4°C. Varying amounts of Con-A (final concentration 60 nM to 40 μM) or normal saline were then added and the entire reaction was incubated for an additional 24 hours at 4°C. (Unless specified receptor or AChR refers to hydroxylapatite purified receptors.) Samples from each reaction tube were assayed on Con-A or HTP columns, or the Con-A/HTP cascade. Nonspecific binding was determined by adding unlabelled toxin (final concentrations 1 μM) to duplicate samples 2 hours prior to the addition of labelled toxin.

2. Binding of labelled and unlabelled AChR to Concanavalin-A-Sepharose and Lens culinaris-Agarose affinity columns

Duplicate samples of AChR (final concentration 2 nM) were labelled with ^{125}I-α-BuTx (final concentration 20 nM) for 24 hours at 4°C. Aliquots from each sample were assayed on 0.2 ml columns of Concanavalin-A-Sepharose of Lens culinaris-Agarose. Non-specific binding was determined as previously described.

An amount of unlabelled AChR equivalent to the amount of labelled receptor actually applied to the above described Con-A or LCA columns was applied to 0.2 ml columns of Con-A or LCA in a volume of 65 μl (one void volume of the columns). The receptors were allowed to equilibrate with the column for 2 hours and were labelled with a four fold excess of ^{125}I-α-BuTx in 65 μl of Buffer B. Following 24 hours at 4°C, the columns were washed with 4 ml of 5 mM $PO_4^{\equiv}$ (pH = 7.4),

150 mM NaCl, 1% Triton X-100 to remove unbound toxin. Nonspecific binding was determined by applying a 200 fold excess of unlabelled toxin to another pair of similarly treated Con-A or Lens columns in a volume of 65 µl 2 hours prior to the addition of labelled toxin.

A toxin blank was run in parallel with the above assays. 65 µl of Buffer B was applied to a Con-A or LCA Column and ^{125}I-α-BuTx was applied as described above. Following 24 hours at 4°C, the columns were washed with 4 ml PBS/1% and counted.

3. The binding of ^{125}I-α-BuTx to AChR: effects of incubation with Con-A

Duplicate samples containing varying concentrations of Con-A (final concentration 50 nM to 25 µM) were preincubated with AChR (final concentration 3 nM) for 24 hours at 4°C. Labelled α-BuTx was added (final concentration 20 nM) and the entire reaction was incubated for an additional 24 hours at 4°C. Nonspecific binding was determined by adding unlabelled α-BuTx (final concentration 6 µM) to duplicate samples at each Con-A concentration 2 hours prior to the addition of labelled toxin. Duplicate 25 µl samples from each tube were assayed using the HTP method. Duplicate 15 µl samples from each tube were immunoprecipitated with goat anti-Con-A and rabbit anti-goat antiserum as previously described. The entire process was repeated reversing the order of the addition of Con-A and ^{125}I-α-BuTx.

4. Kinetics of ^{125}I-α-BuTx binding to AChR: effects of preincubation of AChR with Con-A

The binding reaction mixture contained in a volume of 2.25 ml: 3.3 nM AChR, 1% Triton X-100, 50 mM $PO_4^{\equiv}$ buffer (pH = 7.4), 0.3 mM EDTA, and 0.01% NaN_3. The Con-A treated receptors contained 19.5 µM

Con-A while the control receptors contained normal saline. The recepotrs were preincubated in the above media for 24 hours at 4°C prior to the addition of ^{125}I-α-BuTx (final concentration 10.95 nM). Nonspecific binding of labelled toxin was determined by adding unlabelled toxin to a similar reaction mixture 2 hours prior to the addition of the labelled toxin.

The binding reaction was stopped at various times after the addition of the labelled toxin by transferral of a 60 µl sample to a plastic stoppered tube containing a 200 fold excess of unlabelled toxin. Triplicate samples of 20 µl from each tube were assayed immediately on 1 ml HTP columns. All incubations, binding reactions and assays were performed at 4°C.

The initial concentrations of labelled α-BuTx (B_o) were determined by counting triplicate 20 µl samples taken from the reaction mixtures at 15 and 300 minutes after the addition of the labelled toxin. The initial concentration of AChR (A_o) was determined independently using a saturating concentration of ^{125}I-α-BuTx (35 nM) and assaying the reaction following 24 hours incubation with either Con-A or saline followed by 24 hours incubation with labelled toxin.

5. Apparent equilibrium binding of ^{125}I-α-BuTx to AChR: effects of preincubation of AChR with Con-A

Quadruplicate samples containing AChR (final concentration 1.1 nM) and either Con-A (final concentration 16.6 µM) or normal saline were preincubated for 24 hours at 4°C. Varying amounts of ^{125}I-α-BuTx (final concentration 0.46 nM to 50.4 nM) were added and the reaction was allowed to proceed for 24 hours at 4°C. Nonspecific binding was determined by adding unlabelled toxin (final concnetration

2.4 μM) to quadruplicate samples 2 hours prior to the addition of labelled toxin. Samples from each tube were assayed on 1 ml HTP columns.

6. Binding of ^{125}I-α-BuTx to Lens culinaris purified AChR: effects of incubation with Con-A

Duplicate samples of Lens culinaris purified AChR (final concentration 2.1 nM) were preincubated with varying concentrations of Con-A (0.1 μM to .176 mM) for 24 hours at 4°C. ^{125}I-α-BuTx (final concentration 20 nM) was added and the entire reaction was incubated for an additional 24 hours at 4°C. Nonspecific binding was determined as previously described. Aliquots from each sample were assayed using the HTP column method. The entire procedure was repeated reversing the order of addition of Con-A and ^{125}I-α-BuTx.

7. Kinetics of ^{125}I-α-BuTx binding to Lens culinaris purified AChR

The kinetics of ^{125}I-α-BuTx binding to Lens culinaris purified receptors was determined as previously described for saline and Con-A treated hydroxylapatite purified AChR. The reaction mixture contained 2.6 nM AChR, 0.3 mM EDTA, 0.01% NaN_3, 1% Triton X-100, 50 mM $PO_4^{=}$ (pH = 7.4) and 9.38 nM ^{125}I-α-BuTx. The assay system used in this instance was the HTP method.

8. Dissociation kinetics of AChR ^{125}I-α-BuTx complexes

Duplicate samples of hydroxylapatite or Lens culinaris-Agrose purified AChR (final concentration 2 nM) were labelled for 24 hours at 4°C with ^{125}I-α-BuTx (final concentration 20 nM). A large excess of unlabelled α-BuTx (final concentration 6 μM) was added at time zero, and samples from the reaction mixture were assayed at various

times over a 70 hour period using the HTP column method. Non-specific binding was determined as previously described.

9. Procedure for determining the presence of anti-receptor factors in the serum and immunoglobulin-G fractions of myasthenic patients

Serum (50 µl) or IgG fractions (75 µl) from normal and myasthenic patients were preincubated with AChR (final concentration 2.75 nM) for 18 hours at 4°C. The receptors were then labelled with ^{125}I-α-BuTx (final concentration 20 nM) for an additional 18 hours at 4°C. Each sample was divided for assay on a Con-A column and immunoprecipitation (IgG's only) with goat anti-human IgG or on the Con-A/HTP cascade.

10. Effects of myasthenic IgG containing factor-I on the binding of AChR to the Con-A/HTP cascade and HTP columns

IgG fractions of several myasthenic patients were pooled and concentrated to 25% of their original volume with an Amicon B15 Macrosolute Concentration Cell (MW cutoff = 15,000). The concentration of the resulting IgG solution was determined spectrophotometrically. AChR (final concentration 1.2 nM) was preincubated with varying concentrations of myasthenic IgG containing factor-I (MG-I) (final concentration 0.1 µM to 13.2 µM) for 24 hours at 4°C and then labelled with ^{125}I-α-BuTx (final concentration 15 nM) for 24 hours at 4°C. Nonspecific binding of labelled toxin was determined as previously described. The samples were divided for assay on either the Con-A/HTP cascade and immunoprecipitation or the HTP method and immunoprecipitation. The entire process was repeated with

the order of addition of the IgG and labelled toxin reversed. Controls contained similar concentrations of normal human IgG.

11. Effects of myasthenic IgG containing factor-I on the kinetics of ^{125}I-α-BuTx binding to AChR

The reaction mixture contained 3.3 nM AChR, 1% Triton X-100, 50 mM $PO_4^{=}$ (pH = 7.4), 0.03 mM/EDTA, 0.01% NaN_3 and 10 μM normal or myasthenic IgG containing factor-I in a volume of 2.25 ml. The preincubation, binding reaction, and determination of the initial concentrations of AChR and ^{125}I-α-BuTx were performed as previously described for the Con-A treated AChR.

12. Effects of myasthenic IgG containing factor-S on the binding of AChR to the Con-A and Con-A/HTP cascade columns

AChR (final concentration 3 nM) were preincubated with myasthenic IgG containing factor-S (MG-S) or normal IgG (final concentration 0.83 μM to 90 μM) for 18 hours at 4°C. The samples were assayed by either the Con-A or the Con-A/HTP cascade column method. Nonspecific binding was determined as previously described.

13. Effects of prelabelling AChR prior to exposure to myasthenic IgG containing factor-S on binding of AChR to Con-A/HTP cascade

AChR (final concentration 2.15 nM) were labelled with ^{125}I-α-BuTx for 18 hours at 4°C and then incubated with varying concentrations of MG-S or normal IgG for 18 hours at 4°C. The samples were assayed on the Con-A/HTP cascade. Nonspecific binding was determined as previously described. Another set of samples was assayed in parallel with the above samples, differing only in the order of addition of toxin and IgG.

14. Effects of myasthenic IgG containing factor-S on the kinetics of ^{125}I-α-BuTx binding to AChR

Kinetics of ^{125}I-α-BuTx binding to AChR was determined as previously described for Con-A treated AChR. The reaction mixture contained 3.2 nM AChR, 80 μM normal or myasthenic IgG containing factor-S, 0.3 mM EDTA, 0.01% NaN_3, 1% Triton X-100, 50 mM $PO_4^{\equiv}$ (pH = 7.4), and 9 nM ^{125}I-α-BuTx. The assay system in this instance was the Con-A/HTP cascade rather than the HTP method used in the kinetics experiments previously described.

15. Effects of Protein-A-Sepharose purified myasthenic IgG containing factor-S on the binding of AChR to the Con-A/HTP cascade

IgG fractions were prepared from the sera of 2 patients (M205 and M218), previously found to have factor-S, using the Protein-A-Sepharose affinity chromatography method described in the "Preparations" section of these methods. All fractions eluted from the column were assayed using the Con-A/HTP cascade. Duplicate samples of AChR (final concentration 2.5 nM) were preincubated with MG-S (final concentration 50 μM) for 24 hours at 4°C and labelled with ^{125}I-α-BuTx (final concentration 20 nM) for 18 hours at 4°C. Nonspecific binding was determined as previously described.

16. Effects of myasthenic IgG containing factor-S on the binding of Lens culinaris purified AChR to the Con-A/HTP cascade

Lens culinaris purified AChR (final concentration 2.1 nM) were preincubated with MG-S or normal IgG (final concentration 44 μM) for 18 hours at 4°C, and labelled with ^{125}I-α-BuTx (final concentration 15 nM) for 18 hours at 4°C. The samples were assayed using the Con-A/HTP cascade method. Nonspecific binding was determined as previously

described. The entire procedure was repeated reversing the order of addition of ^{125}I-α-BuTx and IgG.

17. Kinetics of ^{125}I-α-BuTx binding to Lens culinaris purified AChR: effects of preincubation with MG-S

The kinetics of ^{125}I-α-BuTx binding to Lens culinaris purified AChR preincubated with normal IgG or MG-S was determined as previously described for MG-S treated hydroxylapatite purified AChR. The reaction mixture contained 2.8 nM AChR, 80 μM normal or myasthenic IgG containing factor-S, 0.3 mM EDTA, 0.01% NaN_3, 1% Triton X-100, 50 mM $PO_4^{=}$ (pH = 7.4) and 9.5 nM ^{125}I-α-BuTx. The assay system used was the Con-A/HTP cascade.

18. Effects of myasthenic IgG containing factor-B on the binding of ^{125}I-α-BuTx to AChR

Duplicate samples containing AChR (final concentration 1.3 nM) were preincubated with varying concentrations of myasthenic IgG containing factor-B (final concentration 1.0 μM to 0.1 mM) for 18 hours at 4°C. The samples were divided for assay using either the Con-A/HTP cascade or HTP method. Nonspecific binding was determined as previously described. The entire procedure was repeated reversing the order of addition of ^{125}I-α-BuTx and IgG.

19. Apparent equilibrium binding of ^{125}I-α-BuTx to AChR: effects of preincubation of AChR with myasthenic IgG containing factor-B

Quadruplicate samples containing AChR (final concentration 1.1 nM) and either MG-B or normal IgG (final concentration 50 μM) were preincubated for 24 hours at 4°C. Varying amounts of ^{125}I-α-BuTx (final concentration 0.06 nM to 58.7 nM) were added and the reaction

was allowed to proceed for 24 hours at 4°C. Nonspecific binding was determined by adding unlabelled toxin (final concentration 2.4 μM) to quadruplicate samples 2 hours prior to the addition of labelled toxin. Samples were assayed using the HTP column method.

20. Effects of Protein-A-Sepharose purified myasthenic IgG containing factor-B on the binding of ^{125}I-α-BuTx to AChR

Protein-A-Sepharose purified IgG fractions were prepared from the serum of three patients previously found to have MG-B (M108, M174 and M250), as well as the DEAE purified IgG fraction of patient M250. All fractions eluted from the affinity column were assayed for inhibition of toxin binding to AChR. Duplicate samples of AChR (final concentration 1.5 nM) were preincubated with MG-B (final concentration 50 μM) for 24 hours at 4°C, and then labelled with ^{125}I-α-BuTx (final concentration 20 nM) for 18 hours at 4°C.

A dose-response curve was established for the inhibition of toxin binding to AChR preincubated with the Protein-A purified IgG fraction of M174. Duplicate samples of AChR (final concentration 1.5 nM) were preincubated with Protein-A purified MG-B or normal IgG (final concentration 1.2 μM to 35 μM) for 18 hours at 4°C. Nonspecific binding was determined as previously described. The samples were divided for assay by immunoprecipitation and the HTP column method. The procedure was repeated reversing the order of addition of toxin and IgG.

21. Effects of myasthenic IgG containing factor-B on the binding of ^{125}I-α-BuTx to Lens culinaris purified AChR

Lens culinaris purified AChR (final concentration 1.5 nM) were preincubated with MG-B or normal IgG (final concentration 43.9 μM)

for 18 hours at 4°C and labelled with ^{125}I-α-BuTx (final concentration 20.5 nM) for 18 hours at 4°C. Samples were assayed using the HTP column method. Nonspecific binding was determined as previously described. The entire procedure was repeated reversing the order of addition of ^{125}I-α-BuTx and IgG.

22. Interaction of Con-A and myasthenic IgG containing factor-I with labelled AChR

Duplicate samples containing AChR (final concentration 1.5 nM) were labelled with ^{125}I-α-BuTx (final concentration 20 nM) for 18 hours at 4°C. The labelled receptors were incubated for 18 hours at 4°C with either Con-A (final concentration 15 μM), MG-I (final concentration 10 μM), or normal IgG (final concentration 10 μM), followed by another 18 hour incubation at 4°C with either MG-I or normal IgG (final concentration 10 μM) to the samples incubated with Con-A, or Con-A (final concentration 15 μM) to the samples containing MG-I or normal IgG. Controls contained one of the follwoing: normal saline, Con-A (final concentration 15 μM), MG-I or normal IgG (10 μM). Nonspecific binding was determined as previously described. The samples were divided for assay by immunoprecipitation for Con-A and human IgG, and the HTP column method. The entire process was repeated, adding 10 mM α-methyl-D-mannoside following labelling 2 hours prior to the addition of either Con-A or IgG.

23. Interaction of Con-A and myasthenic IgG containing factor-B with AChR: effects on the binding of ^{125}I-α-BuTx to AChR

Triplicate samples containing AChR (final concentration 1.5 nM) were incubated for 18 hours at 4°C with Con-A (final concentration 15 μM), MG-B (final concentration 40 μM) or normal IgG (final con-

centration 40 μM). Another 18 hour incubation followed the addition of MG-B or normal IgG (final concentration 40 μM) to the samples containing Con-A, or Con-A (final concentration 15 μM) to the samples containing MG-B or normal IgG. Control samples contained one of the following: normal IgG or MG-B (final concentration 40 μM), Con-A (final concentration 15 μM), or normal saline. All samples were labelled with ^{125}I-α-BuTx for 18 hours at 4°C and assayed using the HTP column method. Nonspecific binding was determined as previously described. The procedure was repeated using labelled receptors. All samples contained 10 mM α-methyl-D-mannoside, which was added prior to Con-A or MG-B.

D. DATA ANALYSIS

1. Kinetics of α-BuTx binding to AChR

The rate of toxin-receptor complex formation was assumed to follow a second order rate process following the results of Brockes and Hall (1975). The data was analyzed for either one kinetic component or two independent kinetic components present in equal amounts (Brockes and Hall, 1975). The equation used in each case was the integrated form of the second order rate equation as a function of time and the corresponding free ^{125}I-α-BuTx concentration.

Two component equation:

$$\text{AChR-BuTx } (T,B) = A_o\left[1 - \frac{Be^{-k_1T(B_o-A_o)}}{B_o}\right] + C_o\left[1 - \frac{Be^{-k_2T(B_o-C_o)}}{B_o}\right]$$

One component equation:

$$\text{AChR-BuTx } (T,B) = A_o\left[1 - \frac{Be^{-kT(B_o-A_o)}}{B_o}\right]$$

A_o = initial concentration of $AChR_1$ (or total concentration of AChR in the one component model)

C_o = initial concentration of $AChR_2$ in the two component model

B_o = initial concentration of ^{125}I-α-BuTx

B = concentration of α-BuTx at time T

T = time

k_1 = rate constant for the formation of $AChR_1$-α-BuTx complex (or AChR-α-BuTx complex in the one component model)

k_2 = rate constant for the formation of $AChR_2$-α-BuTx

The actual computations were made using the PROPHET[1] computer system curve fitting and non-linear regression programs using the above equations. The concentration of free toxin at any time T was computed by subtraction of the total number of moles of complex formed at time T from initial number of moles labelled toxin at T = 0.

The model which provided the better description of the data was determined using an F-test comparing the residuals (sum of squares) of the best fit lines to the data. The model with a significantly smaller residual ($P < .05$) was considered to provide a better description of the data. Where no significant difference between the two models occurred, the model with the least assumptions, i.e., the one component model, was considered to provide the better description.

2. Data analysis dose-response relationships

All dose-response relationships data was analyzed on the PROPHET computer system using the "Logistic" fitting procedure.[2] This program carries out a least squares fit of the dose-response data using the equation:

$$Y = \frac{Ymax \times A^n}{A^n \times K^n}$$

where Y = response at concentration A

K = concentration of A at which Y = ½ Ymax, i.e., ED_{50}

A = concentration of substance A causing response Y

n = Hill coefficient

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1. The PROPHET System is a national computer resource sponsored by the National Institutes of Health through the Chemical/Biological Information Handling Program, Division of Research.
2. "Public Procedures: A Program Exchange for PHOPHET Users", J.J. Wood, ed., Bolt, Beranek and Newman Inc., 1977, pp. 3-19 to 3-25.

For the present studies, Y is the percent of control binding, Ymax is the amount of binding in the absence of either Con-A or M.G. IgG (taken as 100%) and A is the concentration of either Con-A or IgG.

3. Apparent equilibrium binding of ^{125}I-α-BuTx to AChR

Equilibrium binding data was analyzed using the "Binding" program developed by Dr. D. Edwards[3] for use with the PROPHET computer system. Basically, this program evaluates the binding data using the hyperbolic binding equation:

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

where Y = concentration of AChR- ^{125}I-α-BuTx complex formed at concentration A of α-BuTx

K_D = apparent dissociation constant for ^{125}I-α-BuTx

A = concentration of ^{125}I-α-BuTx

The program determines the K_D and the maximum amount of toxin-receptor formed, and plots the data in both hyperbolic and double reciprocal form.

The equilibrium binding data was also analyzed by the method of Scatchard (1949). The results of this analysis were found to agree excellently with the computer results for Ymax and K_D.

4. Dissociation kinetics of AChR- ^{125}I-α-BuTx complexes

The data for the dissociation of ^{125}I-α-BuTx- AChR complexes were analyzed using the "Expfit" curve fitting procedure of the PROPHET computer system[4]. The Expfit program allows one to fit weighted

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3. Department of Pharmacology, Mount Sinai School of Medicine.
4. "Public Procedures: A Program Exchange for PROPHET Users", J.J. Wood, ed., Bolt, Beranek and Newman, Inc., 1977, pp. 3-1 to 3-13.

or unweighted data with a function of the form

$$Y = Ae^{aX}$$

for up to four exponential terms using an iterative regression process.

The data for hydroxylapatite purified AChR were assumed to fit a model of two independent first order dissociation processes (Brockes and Hall, 1975; Raftery et al., 1975). The equation used was

$$Y = Ae^{-k_{-1}T} + Ce^{-k_{-2}T}$$

where

Y = concentration of toxin-receptor complex at time T

T = time (hours) following addition of unlabelled α-BuTx

A = initial concentration of $AChR_1$-α-BuTx complex

k_{-1} = dissociation rate constant corresponding to $AChR_1$-α-BuTx complexes

C = initial concentration of $AChR_2$-α-BuTx complex

k_{-2} = dissociation rate constant corresponding to $AChR_2$-α-BuTx complexes

The program is given the total concentration of complex (Y) at time T and determines A, k_{-1}, C and k_{-2} as well as the coefficient of non-linear correlation (r^2) between the data points and the fitted regression line. The data was weighted using the inverse of the variance of Y found in three experiments.

Dissociation of LCA purified receptor-toxin complexes was analyzed in a similar manner assuming a homogeneous population of receptors. The equation used was

$$Y = Ae^{-k_{-1}T}$$

where

Y = concentration of toxin-receptor complex at time T

T = time (hours) following addition of unlabelled α-BuTx

A = initial concentration of LCA purified receptor-toxin complexes

k_{-1} = dissociation rate constant

IV. RESULTS

A. RECEPTOR PREPARATIONS AND ASSAYS

1. Purification of Triton X-100 solubilized AChR with spheroidal hydroxylapatite chromatography and Lens culinaris-Agarose affinity chromatography

Purification of AChR using hydroxylapatite removes 33% to 50% of the protein found in the crude receptor preparation (determined spectrophotometrically at 280 nm) with a yield of 60% to 90%. Fractions two and three eluted from the hydroxylapatite column with Buffer B contained 50% to 70% and 10% to 20%, respectively, of the AChR applied to the column. No receptors were found in the void volume following application of the crude AChR to the column, nor in any of the Buffer A fractions or any of the other Buffer B fractions. The average concentration of the resulting receptor preparations was 16.45 ± 7.8 nM toxin binding sites.

Further purification of AChR eluted from hydroxylapatite using Lens culinaris Agarose (LCA) affinity chromatography results in a yield of 52.0 ± 7.5% while removing 98.9 ± 2% of the protein found in the hydroxylapatite prepared AChR. Approximately 45% of the AChR were eluted from the hydroxylapatite column of the LCA-hydroxylapatite cascade in the second Buffer B fraction. The remaining receptors (7%) were eluted in the third Buffer B fraction. No significant amount of AChR was found in any of the Buffer A or other Buffer B fractions, nor in the mannose fraction recirculated through the LCA/spheroidal hydroxylapatite cascade. Labelling the LCA column with ^{125}I-α-BuTx revealed that the lectin column retained 45.0 ± 7.5% of the receptors.

The receptors eluted from the hydroxylapatite and LCA columns are characterized in terms of their interactions with α-BuTx, Con-A and myasthenic IgG in the following sections. Unless specifically noted "receptor" or "AChR" refers to hydroxylapatite purified receptors.

2. Binding of labelled AChR to Con-A, HTP and Con-A/HTP cascade columns: effects of incubation of labelled AChR with Con-A

Labelled receptors bind to Con-A Sepharose affinity columns and adsorb to HTP columns. The Con-A column assay data closely parallels that reported by Mittag et al. (1976). However, when HTP adsorption is used approximately 15% more labelled AChR are present compared to the Con-A affinity column alone (Table 1). In the Con-A/HTP cascade method 85% to 95% of the labelled receptors binds to the Con-A affinity column and an additional 5% to 15% binds to the HTP column. Thus, the ratio of Con-A to HTP binding varies slightly from one receptor preparation to another. The amount of labelled receptor that binds to the HTP column is not significantly different from the total amount of receptor found in the cascade system (Con-A plus HTP column).

Binding of labelled AChR to Con-A Sepharose was antagonized in a dose dependent manner by preincubation with soluble Con-A. The Con-A complexed receptors which were blocked from binding to the Con-A column were completely recovered on the HTP column. Thus, Con-A had no effect on the binding of prelabelled receptors to HTP columns. The maximal inhibition of receptor binding the Con-A columns by soluble Con-A was 93.3 ± 1.13% (ED_{50} = 0.53 μM, Hill coefficient = 2.3 ± 0.1) (Fig. 1).

Table 1

Comparison of Concanavalin-A, Hydroxylapatite and Concanavalin-A/ Hydroxylapatite Cascade Column Assays

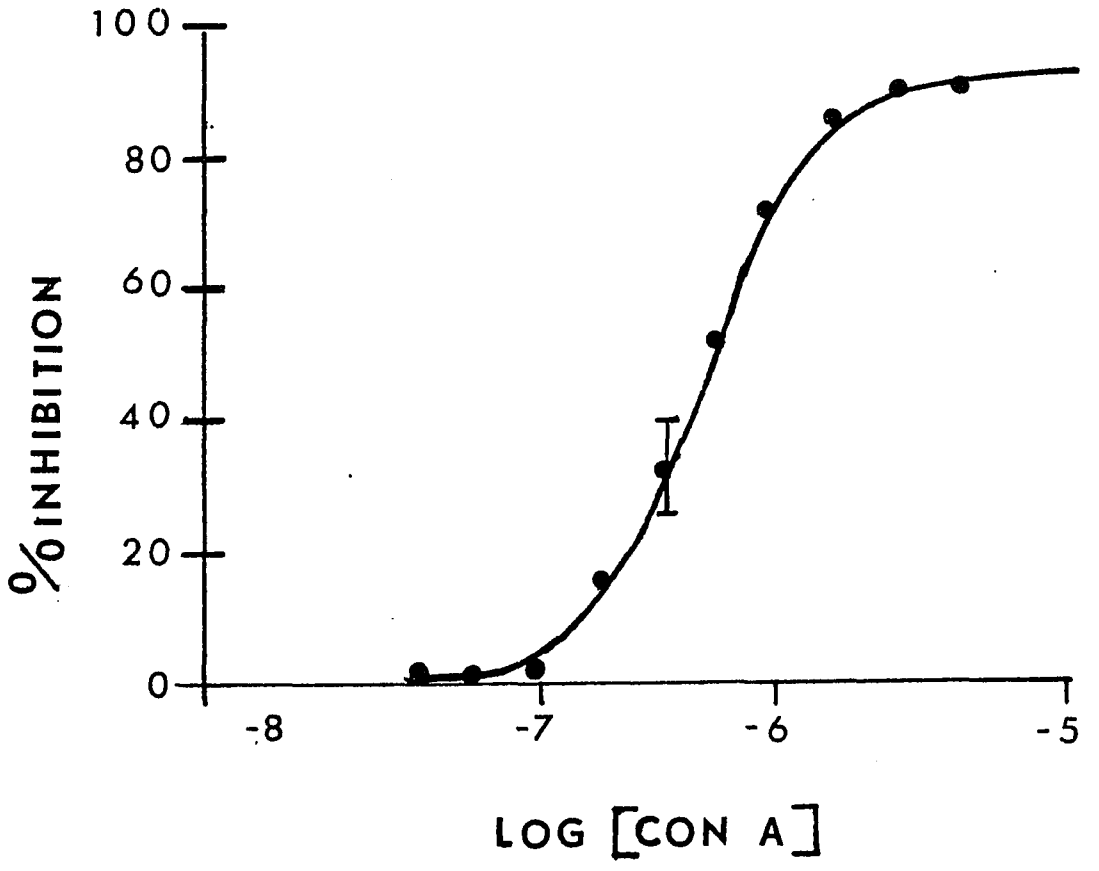
Assay System		α-BuTx-AChR Complex Retained	
		fmoles ± S.D.	(N)
Con-A		179 ± 10.3*	(6)
HTP		204 ± 15.3	(6)
Con A/HTP	Con-A	176 ± 11.9	(6)
	HTP	31 ± 1.9	(6)
		207 ± 13.3	

* The results of Con-A assays were found to be significantly different ($P < .01$, using the two tailed Student's t-test) from those of both the HTP and Con-A/HTP assays. The results of the HTP assay were found not to be significantly different from those of the Con-A/HTP cascade assay ($>> .05$).

Figure 1. Data points are the average for three experiments using three different receptor preparations. The bar (I) represents the average standard deviation of the data points. Computer analysis of these data showed the maximum inhibition to be 93.3 ± 1.13%, (ED_{50} = 0.53 ± 0.01 μM Con-A, Hill coefficient = 2.3 ± 0.1).

Figure 1

Inhibition of Binding of Labelled Receptors Treated with Soluble Con-A to Con-A-Sepharose Affinity Columns



3. Binding of labelled and unlabelled AChR to Concanavalin-A-Sepharose and Lens culinaris-Agarose affinity columns

Labelled and unlabelled receptors bind to both Con-A Sepharose and LCA affinity columns (Table 2). For both labelled and unlabelled receptors, the Lens column bound 15% less AChR than the Con-A column. Binding of free labelled toxin to both columns was negligible.

Table 2

Binding of Labelled and Unlabelled AChR to Lens Culinaris-Agrose and Concanavalin-A-Sepahrose Affinity Columns

	Con-A-Sepharose f-moles bound ± S.D. (N)	Lens-Culinaris-Agrose f-moles bound ± S.D. (N)
Labelled	106 ± 10 (3)	91 ± 7 (3)
Unlabelled	118 ± 7 (3)	103 ± 8 (3)

The binding of labelled or unlabelled AChR to Con-A-Sepharose was found not to be significantly different than that binding to Lens culinaris-Agarose ($p > .05$).

B. EXPERIMENTAL

1. Binding of ^{125}I-α-BuTx to AChR: effects of incubation with Con-A

Concanavalin-A, when incubated with AChR for 24 hours prior to the addition of ^{125}I-α-BuTx, produced a dose dependent inhibition of toxin binding to the receptors. Using the HTP assay system, the maximum inhibition was 41% ± 1.4% with an ED_{50} of 1.00 ± 0.08 μM and a Hill coefficient of 1.9 ± 0.3 (Fig. 2). The remaining receptors which did label with toxin also formed complexes with Con-A, as shown by immunoprecipitation with anti-Con-A which was performed in parallel with the HTP assays. The maximum amount of Con-A blocked receptors precipitated was 67.2 ± 4.4% (ED_{50} = 0.85 ± 0.04 μM, Hill coefficient = 4.3 ± 0.7).

No inhibition of toxin binding was observed when receptors were labelled prior to the addition of Con-A (Fig. 3). Immunoprecipitation of receptor-toxin-Con-A complexes with anti-Con-A antiserum showed that the prelabelled receptors bind Con-A, as evidenced by the precipitation of 93.8 ± 3.1% of the receptors (ED_{50} = 0.79 ± 0.05 μM, Hill coefficient = 5.5 ± 0.7). Note that the only significant difference between the immunoprecipitation curves of pre- and post-labelled AChR is the maximum percent of the receptors precipitated (Fig. 2 and 3).

2. Kinetics of ^{125}I-α-BuTx binding to AChR: effects of preincubation of AChR with Concanavalin-A

The data for the kinetics of toxin binding to AChR preincubated in normal saline or normal IgG was fitted to both one and two component systems, as described in Methods. The two component system

Figure 2. Duplicate samples of AChR (3nM) were preincubated with varying concentrations of Con-A for 24 hours at $4^{o}C$. A second 24 hour incubation followed the addition of ^{125}I-α-BuTx (20nM). Each sample was assayed using the HTP column method as well as immunoprecipitation by anti-Con-A. Data points represent the average of four experiments using four different receptor preparations. The maximum amount of AChR-Con-A complex immunoprecipitated (closed squares) was 67.2 ± 4.4% (ED_{50} = 0.85 ± 0.04 μM, Hill coefficient = 4.3 ± 0.7). The percent AChR-toxin complex recovered following maximal Con-A-inhibition of toxin binding (circles) was 59 ± 1.4% (ED_{50} = 1.00 ± 0.08 μM, Hill coefficient = 1.9 ± 0.3).

Figure 2

Incubation of unlabelled receptors with soluble Con-A

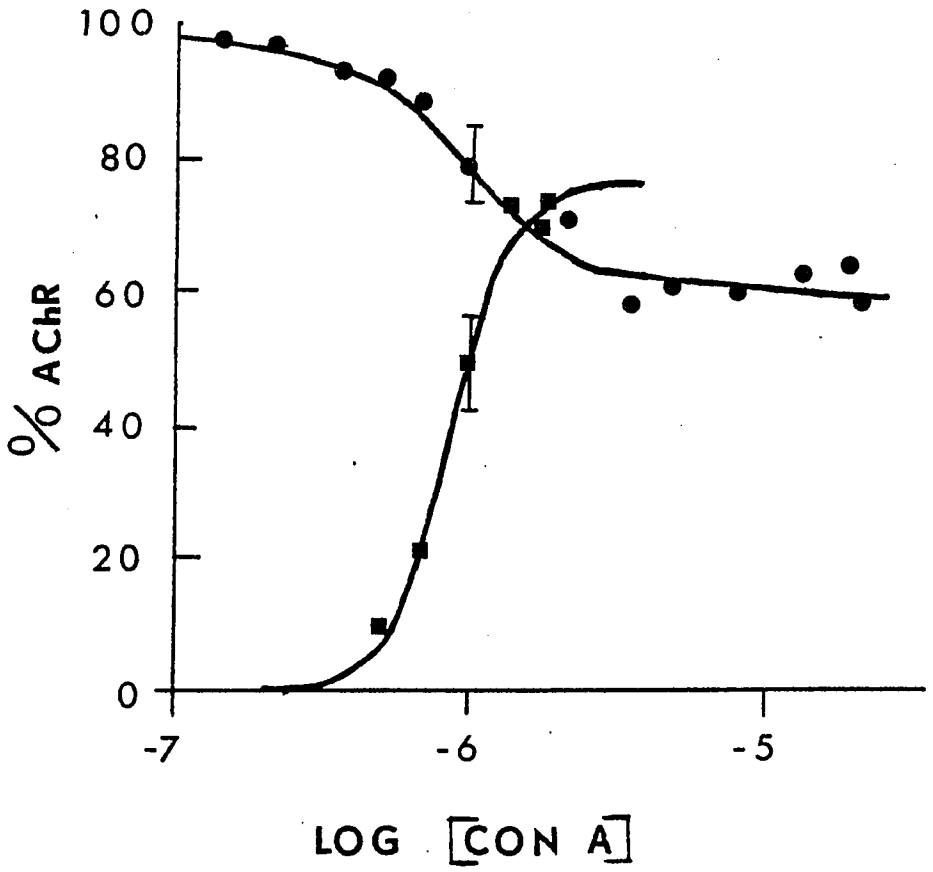
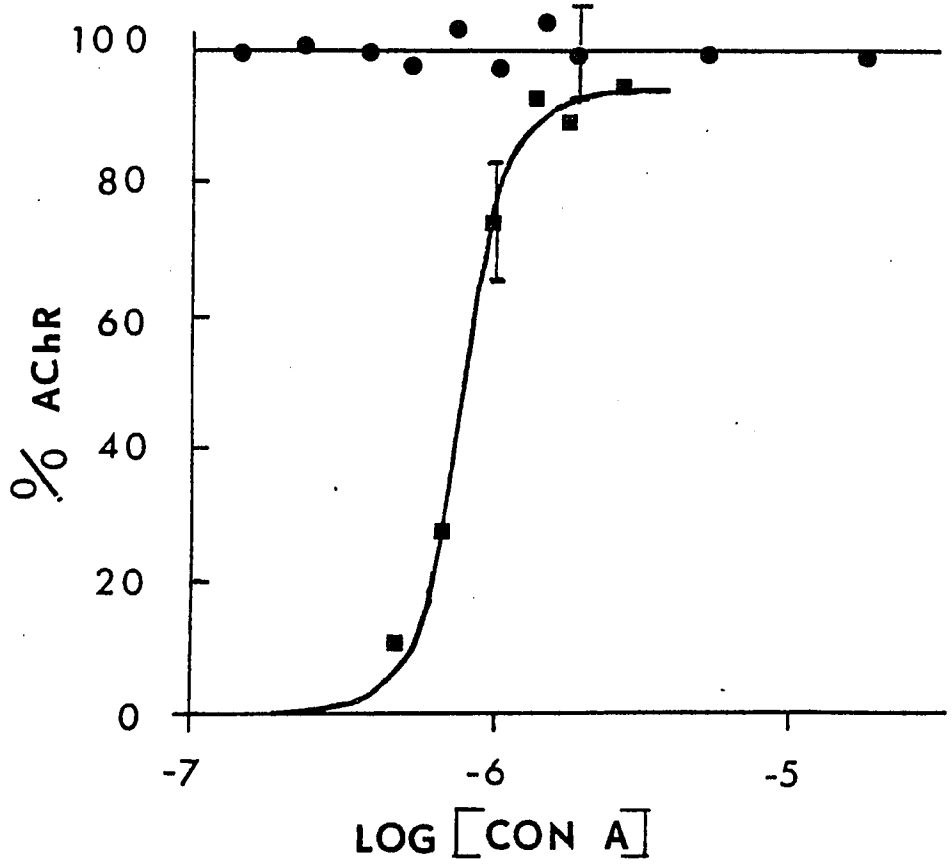


Figure 3. Duplicate samples of AChR (3nM) were labelled for 24 hours at 4°C with ^{125}I-α-BuTx (20nM). A second 24 hour incubation followed the addition of varying concentrations of Con-A. Each sample was assayed using the HTP column method as well as immunoprecipitation by anti-Con-A. Data points represent the average of four experiments using four different receptor preparations. The maximum amount of AChR-Con-A complex immunoprecipitated (squares) was 93.8 ± 3.1% (ED_{50} = 0.79 ± 0.05 μM, Hill coefficient = 5.5 ± 0.7). No dissociation of toxin-receptor complex (circles) was observed as measured by the HTP assay method.

Figure 3

Incubation of labelled receptors with soluble Con-A



(see page 52 of Methods), provided a significantly better fit to the data in both cases than did the one component system (Table 3). The average values for the two rate constants from six experiments are $k_1 = 8.23 \pm 0.8 \times 10^4 M^{-1}s^{-1}$ and $k_2 = 0.35 \pm 0.04 \times 10^4 M^{-1}s^{-1}$ (Fig. 4).

Following pretreatment of AChR with a dose of Con-A producing maximal inhibition, toxin binding was inhibited 42% relative to the saline controls (Fig. 5). The kinetics of toxin binding to the remaining receptors (Con-A-receptor complexes) could be equally well described by either the one component or the two component model. Therefore, the simplest case is that toxin binding to the remaining receptors fits the one component model. The rate constant for the binding of toxin to these receptors is $0.35 \pm 0.02 \times 10^4 M^{-1}s^{-1}$. This value corresponds to the slow toxin binding component found for the saline treated receptors.

3. Apparent equilibrium binding of ^{125}I-α-BuTx to AChR: effects of preincubation of receptors with Con-A

The results of equilibrium binding of ^{125}I-α-BuTx to AChR preincubation in saline are shown in Fig. 6. Analysis of this data using the method of Scatchard (1949) indicates the presence of a homogeneous population of α-BuTx binding sites with an apparent dissociation constant, K_{Dapp}, of 0.72 ± 0.18 nM (Fig.7).

The apparent K_D of that fraction of receptors whose toxin binding capacity is unaffected by Con-A (i.e., Con-A-AChR complexes) was 1.7 nM (Fig. 6) and the Scatchard analysis of this data again resulted in a straight line (Fig. 7). The increase in K_{Dapp} is significant ($P < 0.01$). The amount of toxin binding found in the Con-A treated receptors was decreased by 37% relative to the saline controls,

Table 3. *The model providing a better description of the data was determined using an F-test comparing the residuals (sum of squares) of the best fit lines to the data. The two component model provided a better description of the data for HTP prepared AChR treated with normal saline, control IgG, MG-S and Mg-I ($P < .01$). In each case, the values of the two rate constants were significantly different ($P < .01$, using a two-tailed Students's t-test). For Con-A treated HTP-AChR and LCA purified AChR treated with normal saline or control IgG, no significant difference between the two models was observed and the one component model was considered to provide the better description of these data. Models are described in Data Analysis section of Methods, page 52.

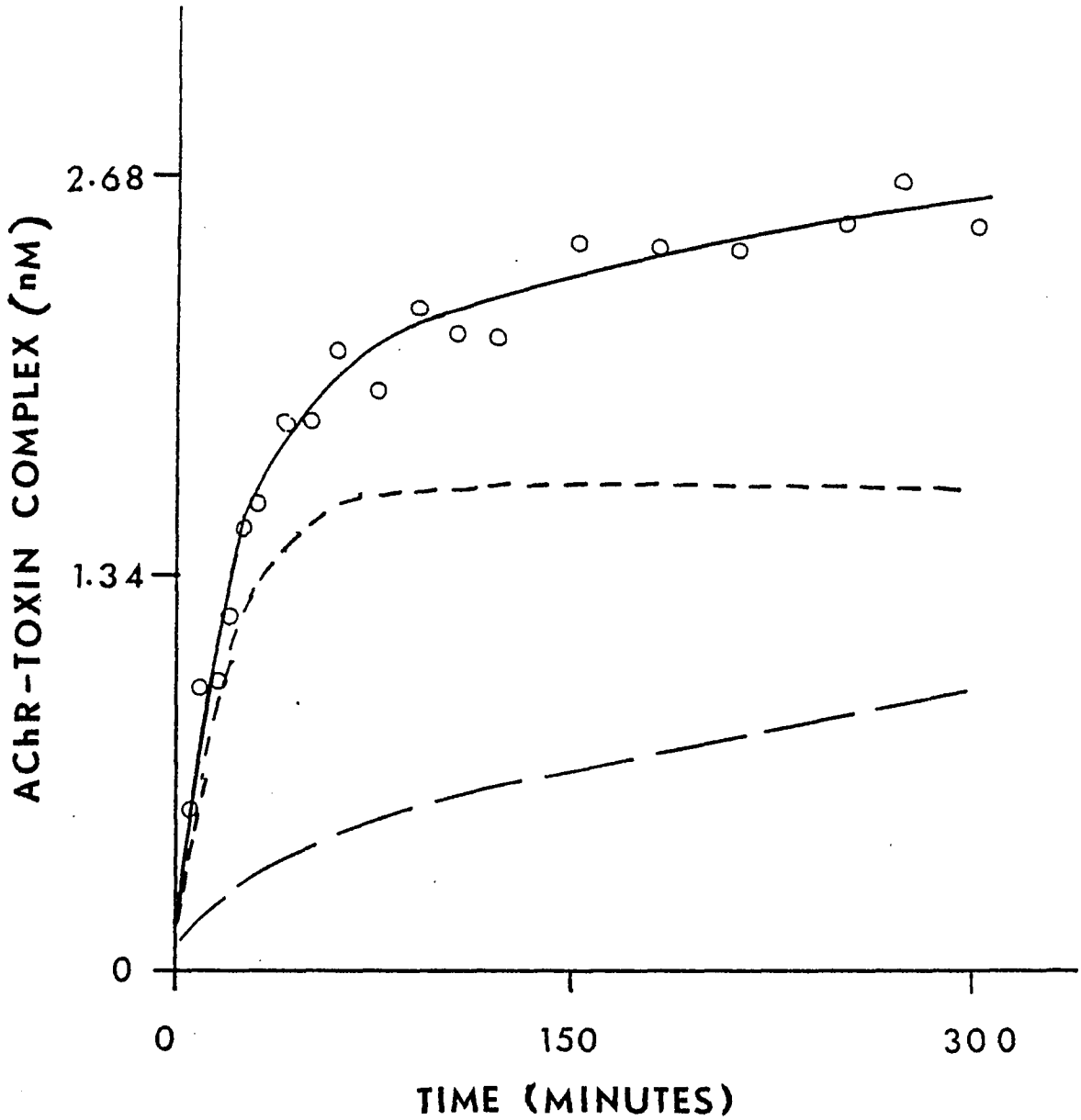
Table 3

Kinetics of ^{125}I-α-BuTx Binding to AChR: Comparison of One versus Two Component Models*

Incubation Medium	Two Component Model			One Component Model	
	$k_1 x 10^{-4} (M^{-1}s^{-1})$	$k_2 x 10^{-4} (M^{-1}s^{-1})$	sum of squares x 10^{20}	$kx10^{-4} (M^{-1}s^{-1})$	sum of squares x 10^{20}
HTP-AChR: Normal Saline or Control IgG (N=6)	8.23 ± 0.7	0.35 ± 0.04	17.7	2.03 ± 0.3	337
Con-A (N=3)	0.56 ± 0.1	0.15 ± 0.03	12.4	0.35 ± 0.02	14.1
MG-S (N=3)	6.43 ± 0.97	0.11 ± 0.01	6.97	1.34 ± 0.29	65.4
MG-I (N=3)	8.26 ± 0.9	0.3 ± 0.03	20.0	1.86 ± 0.1	325
LCA AChR: Normal Saline or Control IgG (N=3)	14.1 ± 3.1	8.56 ± 2.5	8.01	11.8 ± 1.0	8.8
MG-S (N=3)				7.97 ± 0.41	

Figure 4. In this typical experiment the two component model provided a significantly better description of the data. Computer analysis of the data showed $k_1 = 8.2 \times 10^4 M^{-1}s^{-1}$ and $k_2 = 0.37 \times 10^4 M^{-1}s^{-1}$. Similar results were obtained in five other experiments using three different preparations of HTP prepared AChR (see Table 3).

Figure 4



Kinetics of ^{125}I-α-BuTx Binding to AChR

Incubated in Control IgG or Normal Saline

Figure 5. The kinetics of toxin binding to AChR pretreated with normal saline (open triangles) and its resolved slow component (dashed line), described in Fig. 4, are compared to the kinetics of toxin binding to AChR pretreated with 19.5 μM Con-A for 24 hours at $4^{o}C$. Toxin binding to Con-A treated AChR (closed triangles) is best described by a single rate constant, which in this typical experiment was $0.37 \pm 0.02 \times 10^{4} M^{-1} s^{-1}$. The final concentration of AChR-toxin complexes was 1.914 nM, or 58% of the control value. Similar results were obtained in two other experiments using two other AChR preparations (see Table 3).

Figure 5

Kinetics of ^{125}I-α-BuTx binding to AChR preincubated with Con-A

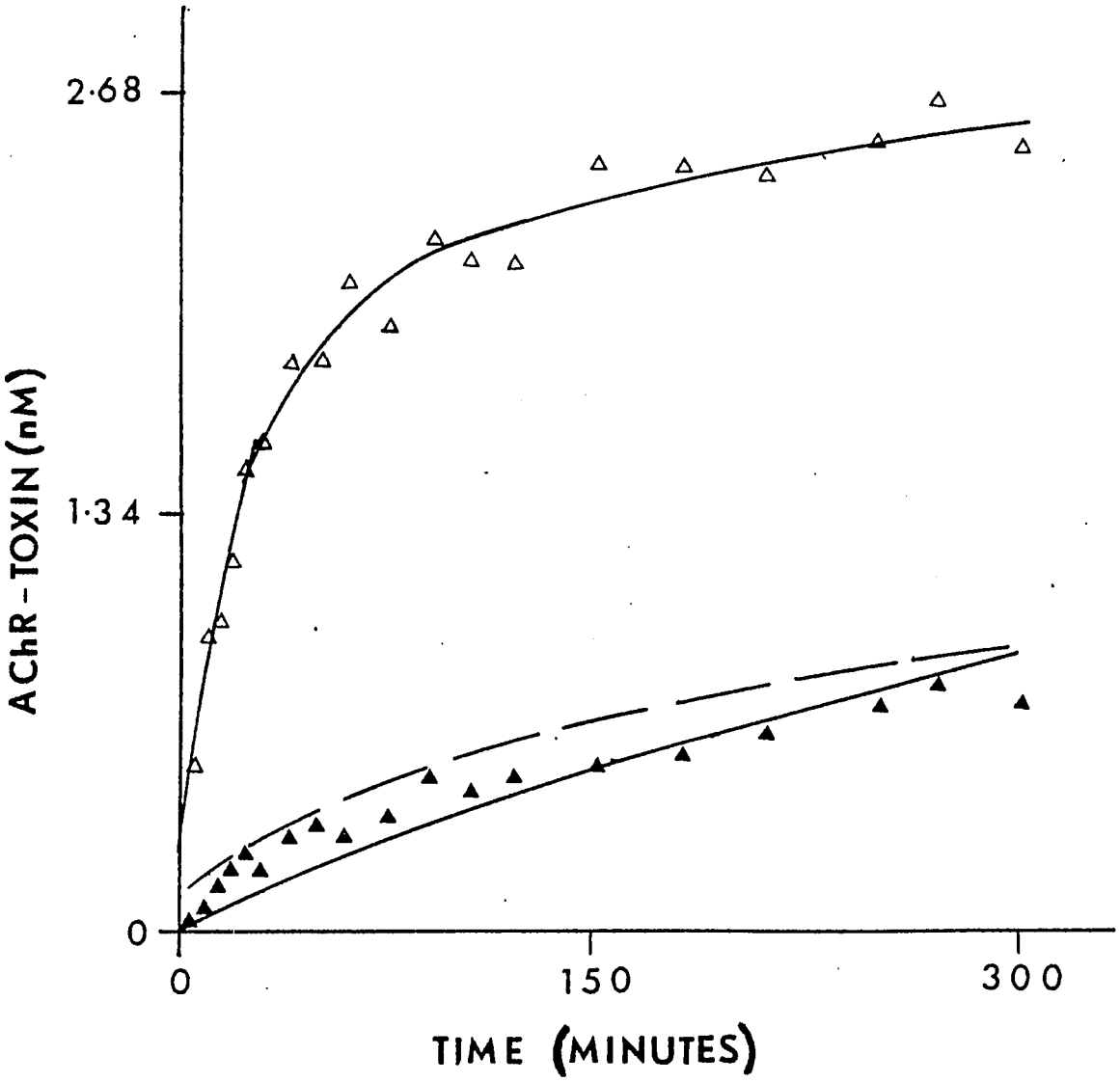


Figure 6. Quadruplicate samples containing AChR and either Con-A or NaCl were preincubated for 24 hours at $4^{o}C$ prior to labelling for 24 hours with varying concentrations of ^{125}I-α-BuTx. All samples were assayed using the HTP column method. In this typical experiment, the K_{Dapp} was 1.7 nM for Con-A treated AChR (closed diamonds) and 0.6 nM for control AChR (open diamonds). Toxin binding to Con-A treated AChR was 63% relative to control. Similar results were obtained in two other experiments using two different receptor preparations.

Figure 6

Apparent Equilibrium Binding of ^{125}I-α-BuTx

to AChR Preincubated with Con-A or Saline

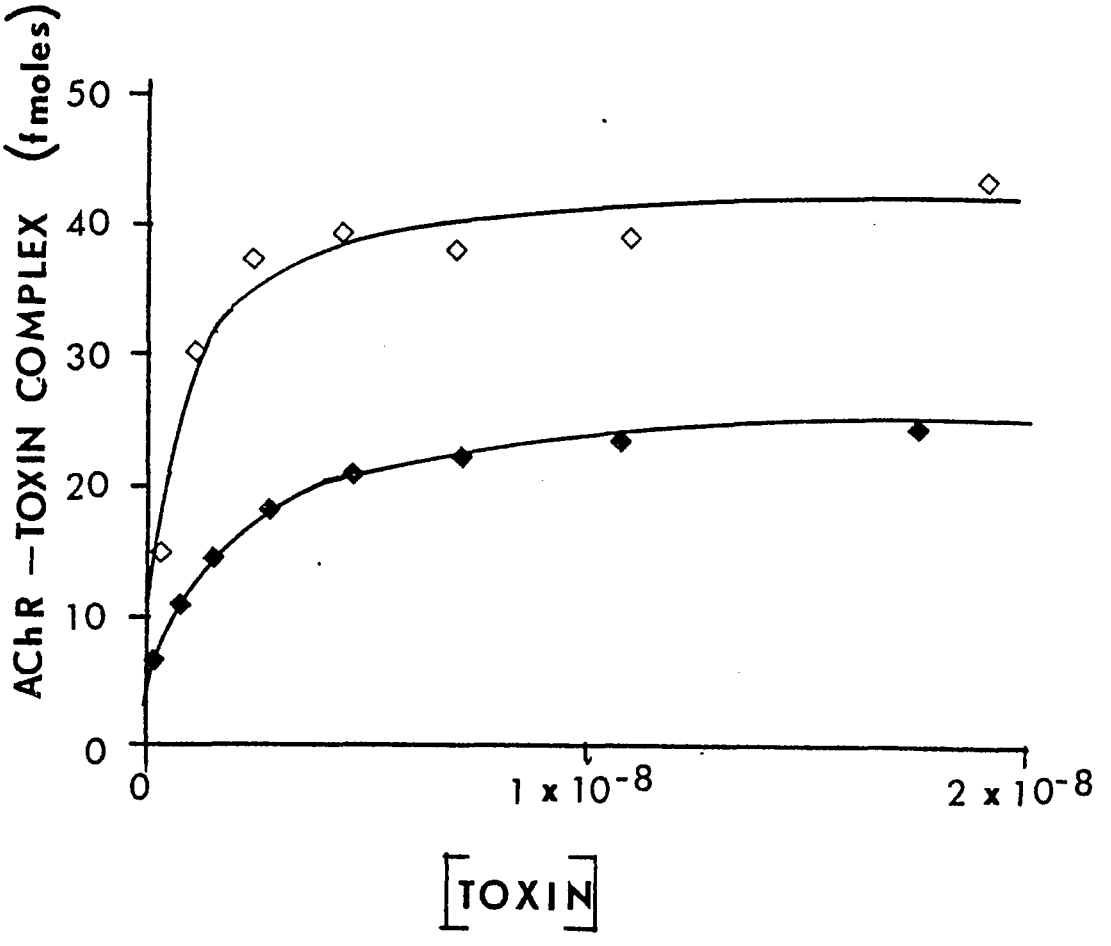
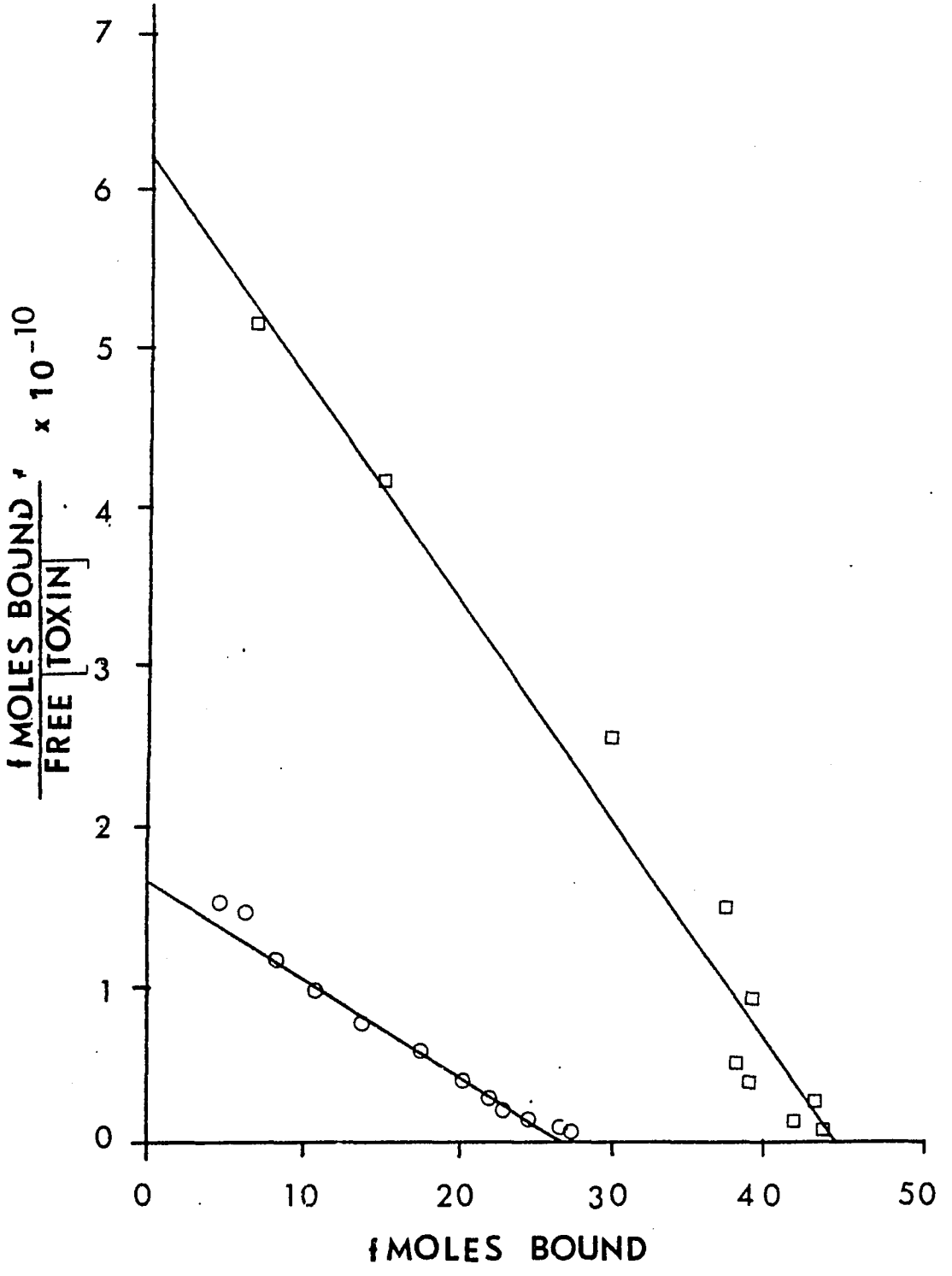


Figure 7. Scatchard analysis of the data presented in Fig. 6 shows that AChR preincubated with normal saline (open squares) or Con-A (open circles) both appear to be homogeneous populations of toxin binding sites. The mean K_{Dapp} for Con-A treated AChR 1.71 ± .07 nM was found to be significantly different ($P < .01$) than the mean K_{Dapp} for control AChR (.72 ± .18 nM).

Figure 7

Equilibrium Binding of Toxin to AChR Incubated with Con-A or Saline



in agreement with the previous experiments showing a 41% inhibition of toxin binding by Con-A pretreatment.

4. Binding of ^{125}I-α-BuTx to LCA purified AChR: effects of incubation with Con-A

Concanavalin-A completely inhibited the binding of toxin to Lens culinaris purified AChR (Fig. 8). The concentrations of Con-A necessary to effect a dose-dependent inhibition of toxin binding parallel to those used to inhibit binding to HTP purified AChR. The ED_{50} and Hill coefficients are essentially the same for both Lens and HTP purified receptors (1.02 ± .05 μM vs 1.00 ± 0.8 μM, and 1.4 ± 0.2 vs 1.9 ± 0.3, respectively). The maximum percent inhibition of toxin binding is 41% of the hydroxylapatite prepared receptors, and 100% for the LCA purified AChR.

5. Kinetics of ^{125}I-α-BuTx binding to LCA purified AChR

The data for the kinetics of toxin binding to LCA purified AChR was fitted to both the one and two component systems. No distinction between the two models can be made using the sum of squares as an indication of which model best describes the data (Table 3). In addition, the rate constants determined using the two component model are not significantly different ($P > .05$). Thus, the model with the least assumptions, i.e., the one component system, provides an adequate description of the data. The rate constant for the binding of toxin to LCA purified AChR was 1.18 ± 0.1 x $10^5 M^{-1}s^{-1}$ (Fig. 9).

6. Dissociation kinetics of AChR- ^{125}I-α-BuTx complexes

The dissociation of toxin labelled HTP prepared receptor complexes, like the association, is best described by a two component system

Figure 8. Duplicate samples of HTP or LCA prepared AChR (2.1 nM) were preincubated with varying concnetrations of Con-A for 24 hours at $4^{o}C$. A second 24 hour incubation followed the addition of 20 nM ^{125}I-α-BuTx. Samples were assayed on 1 ml HTP columns. As noted previously, the maximal Con-A inhibition of toxin binding to HTP AChR (closed circles) is 41% (Fig. 2). However, 100% maximal inhibitor of toxin binding is observed for LCA (open circles) purified AChR (ED_{50} = 1.02 ± 0.05 μM, Hill coefficient = 1.4 ± 0.2).

Figure 8

Con-A Induced Inhibition of Toxin Binding

to HTP Prepared and LCA Purified AChR

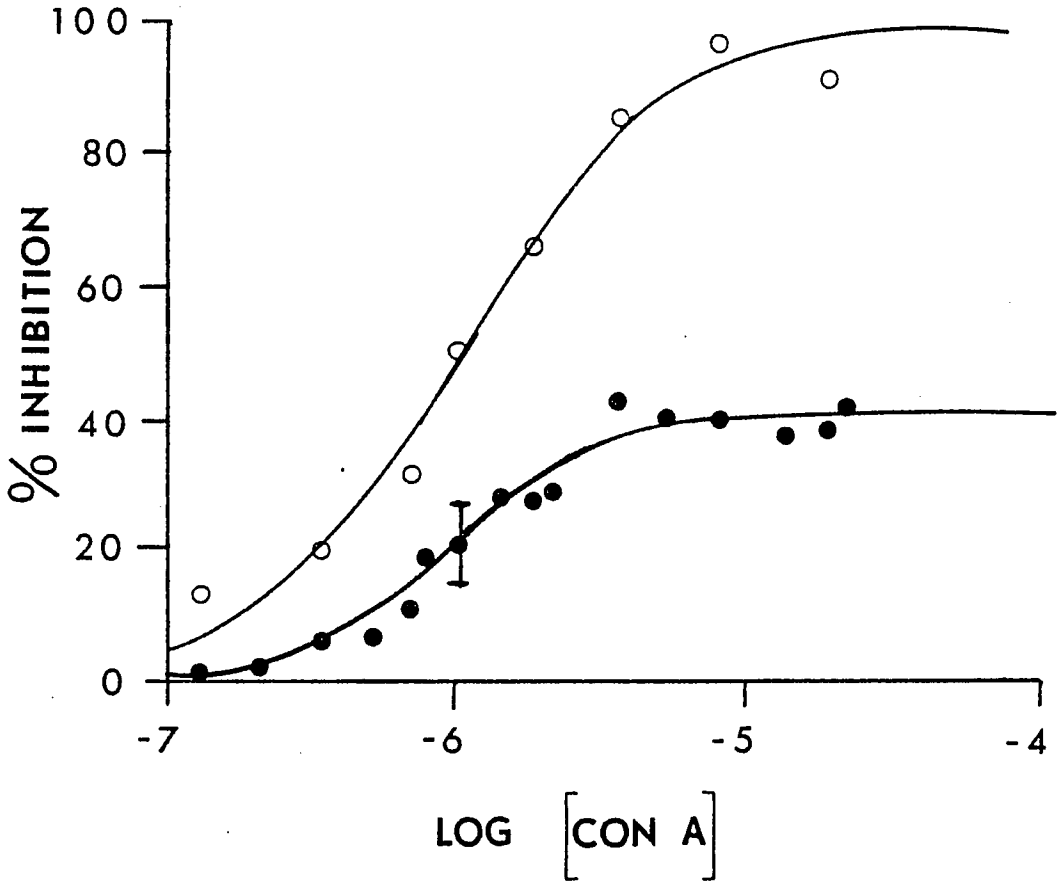
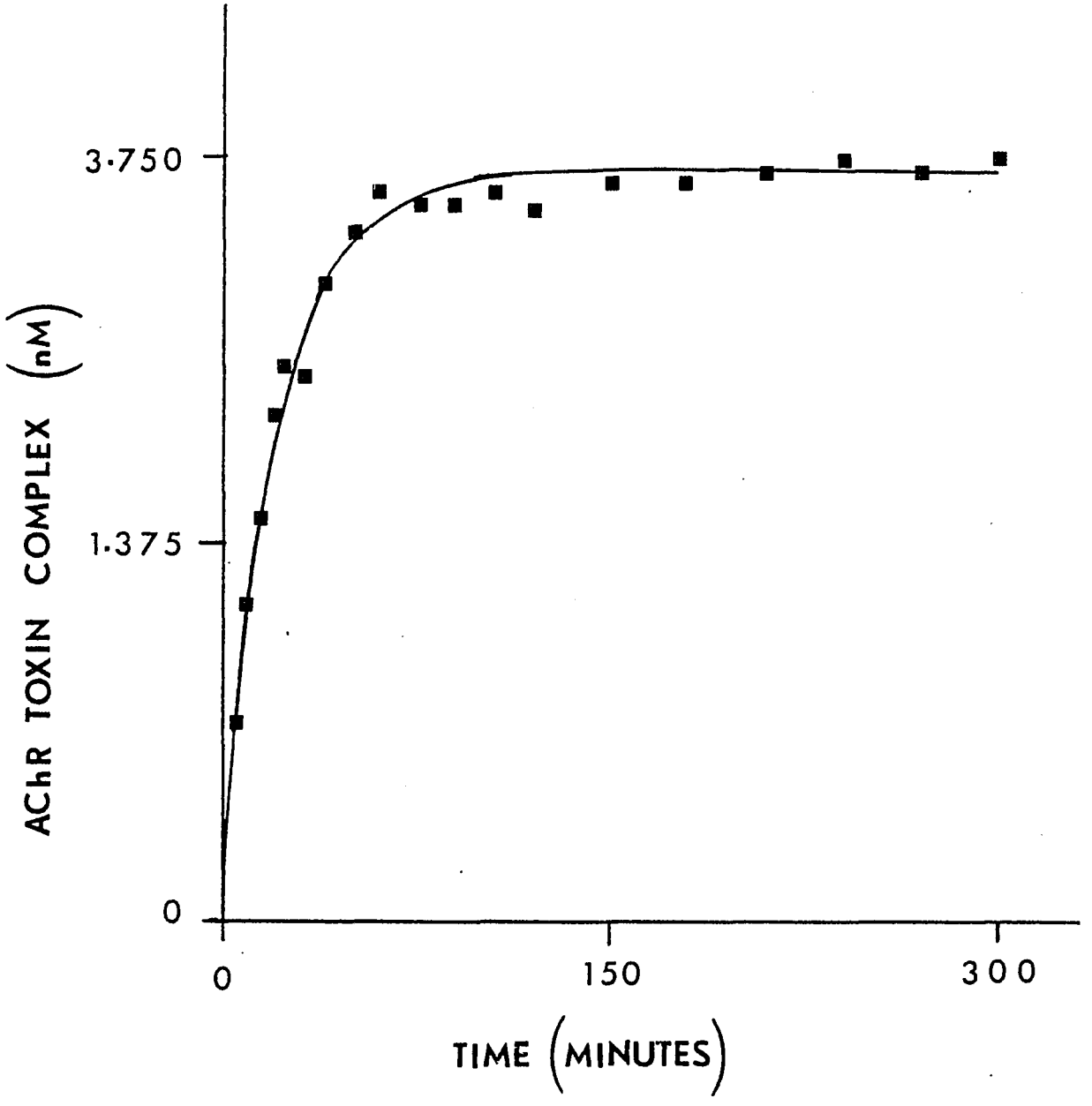


Figure 9. The kinetics of toxin binding to LCA purified AChR in this typical experiment is best described by a single rate constant, $k = 1.21 \times 10^5 M^{-1}s^{-1}$. Similar results were obtained in two other experiments using two different LCA purified AChR preparations (see Table 3).

Figure 9

Kinetics of ^{125}I-α-BuTx Binding to LCA Purified AChR



$$Y = Ae^{-k_{-1}T} + Ce^{-k_{-2}T}$$

A plot of the log of ^{125}I-α-BuTx-AChR complex at time T after the addition of a 300 fold molar excess of unlabelled toxin versus time is not linear (Fig. 10). Computer analysis of the data gives a value of 2.49 ± 0.47 x $10^{-5}s^{-1}$ for k_{-1} and 2.43 ± 0.79 x $10^{-6}s^{-1}$ for k_{-2}. Extrapolation to time zero gives an initial concentration of 1.1 ± 0.14 nM for the first component (corresponding to k_{-1}) and 0.92 ± 0.15 nM for the second component.

The dissociation of LCA purified receptor-toxin complexes occurs with only one rate of dissociation (Fig. 11). The rate constant, as determined by computer analysis, is 3.12 ± 0.12 x $10^{-5}s^{-1}$, corresponding to the fast dissociation rate constant observed for HTP prepared AChR. The initial concentration of complex, estimated by extrapolation to time zero, was found to be 1.95 ± 0.05 nM.

7. Detection of anti-receptor factors in sera and immunoglobulin G fractions of patients with myasthenia gravis

Anti-receptor factors were found in both the sera and DEAE-Sephadex purified IgG fractions of myasthenic patients using either the Con-A or Con-A/HTP cascade column assay. Initial serum screens utilized the Con-A column assay and these results were combined with the Con-A portion of the Con-A/HTP cascade assays. A patient was considered positive for anti-receptor factor if the percent of receptors binding to Con-A was two standard deviations less than that observed for AChR treated with control serum (100 ± 7%), i.e., less than 86% of control (Mittag et al., 1976). Using this criterion 134 of the 220 sera screened (61%) were positive for anti-receptor antibodies. A representative sample of the population tested in this

Figure 10. Duplicate samples of HTP prepared AChR (2nM) were labelled for 24 hours at $4^{o}C$ with 20 nM ^{125}I-α-BuTx. A large excess of unlabelled α-BuTx (6μM) was added at time zero, and samples from the reaction mixture were assayed at various times over a 70 hour period on 1 ml HTP columns. The dissociation of the AChR-α-BuTx complexes is best described by a two component system (see Data Analysis). Computer analysis of these data gives a value of $2.49 \pm 0.47 \times 10^{-5}s^{-1}$ for k_{-1} and $2.43 \pm 0.79 \times 10^{-6}s^{-1}$ for k_{-2} and the initial concentrations of the two forms of AChR-αBuTx complexes were 1.1 ± 0.14 nM and 0.92 ± 0.15 nM respectively. The coefficient of nonlinear regression, r^2, is .998.

Figure 10

Dissociation Kinetics of HTP Prepared AChR-^{125}I-α-BuTx Complexes

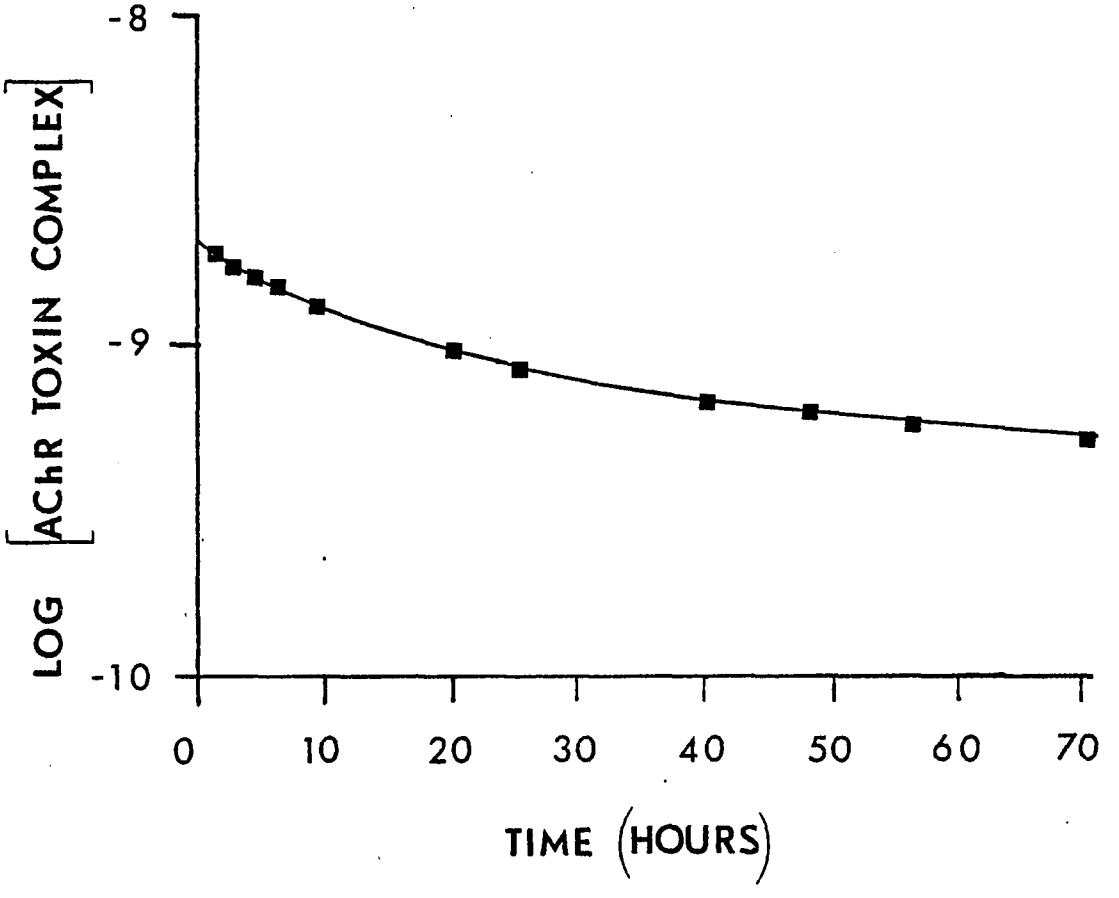
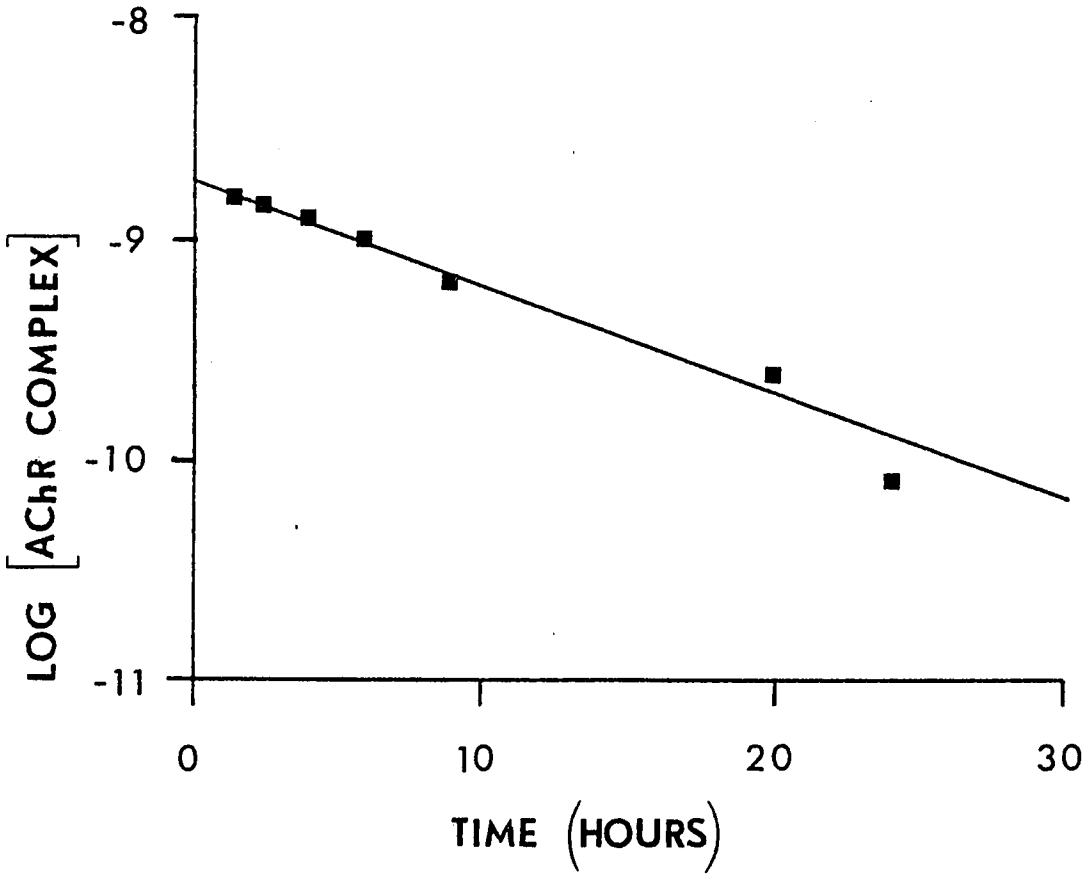


Figure 11. The dissociation of LCA pruified AChR-α-BuTx complexes is best described by a single rate constant (see Data Analysis). Computer analysis of these data gives a value of $3.12 \pm 0.12 \times 10^{-5} s^{-1}$ for k and an initial concentration of AChR-α-BuTx complex of 1.95 ± 0.05 nM, which agrees well with that measured experimentally. The linear correlation coefficient (r^2) is 0.996. Experimental conditions were the same as those described in Fig. 10.

Figure 11

Dissociation of LCA Purified AChR-^{125}I-α-BuTx Complexes



manner appears in Table 4.

Sera tested using the Con-A/HTP cascade assay were considered positive for anti-receptor antibody if either the ratio of Con-A to HTP binding and/or total binding was latered by 1 standard deviation relative to treatment with control serum. The mean ratio of binding for receptor treated with a control normal human serum to Con-A and HTP columns was in the ratio of 3 : 1 and the total binding capacity was 85% ± 19% relative to normal saline. A serum was considered positive for myastheni factor-B (MG-B), i.e., the antibody responsible for inhibition of toxin binding to AChR, if the total amount of receptor bound to the cascade columns was less than 81% of control (see sera 90, 108, 110 and 220 in Table 5). If a serum caused no significant decrease in the total amount of receptor bound to the cascade columns, but decreased the binding ratio between the Con-A and HTP columns from 3:1 to approximately 1:1, the serum was considered positive for MG-S, i.e., that antibody of labelled receptor binding to Con-A-Sepharose (see sera 94, 137, 142, 169 and 205 in Table 5). Note that sera positive for MG-B can also cause an "apparent" decrease of the Con-A/HTP ratio. This decrease occurs without any significant increase in the percent AChR binding to the HTP columns, relative to control (e.g. serum 110, Table 5). Such sera are not considered positive for MG-S. However, sera positive for MG-B and MG-S have been observed (serum 90, Table 5). Such sera cause inhibition of toxin binding as well as a "real" decrease of the Con-A/HTP ratio by inhibition of receptor binding to Con-A-Sepharose. Using these criteria, 121 of 187 sera tested were positive for one or both of these anti-receptor factors. Ninety-one

Table 4

Detection of Anti-Receptor Factors in Myasthenic Sera Using the Con-A Assay

Serum	% Recovery of AChR on Con-A-Sepharose	Anti-Receptor Factor*
M3	80	+
M5	91	-
M6	40	+
M7	80	+
M9	87	-
M10	47	+
M11	52	+
M24	98	-
M24	71	+
M26	75	+
M29	100	-
M30	50	+
M33	81	+
M35	100	-
M50	100	-

*A serum was considered positive (+) for anti-receptor factor if the percent receptors binding to Con-A-Sepharose was two standard deviations less than that observed for AChR treated with control serum (100 ± 7%), i.e., less than 86% of control.

Table 5. *Denotes sera positive for toxin-blocking factor (MG-B).

†Denotes sera positive for Con-A-Sepharose blocking factor (MG-S). See Results, page 90, for description of criteria used to determine presence of toxin-blocking factor (MG-B) or Con-A blocking factor (MG-S).

Table 5

Detection of Anti-Receptor Factors in Myasthenic Sera Using the Con-A/HTP Cascade Assay System

Serum	Con-A-Sepharose Column % AChR	HTP Column % AChR	Con-A/HTP ratio	Total Recovery % AChR
Normal	75 ± 13	25 ± 13	3.00 ± 0.5	100 ± 19
90	33	39†	0.85	72*
94	43	48†	0.90	91
96	58	42	1.38	100
102	61	46†	1.33	107
107	59	35	1.69	94
108	35	25	1.40	60*
110	24	36	0.67	60*
113	46	54†	0.85	100
120	82	36	2.28	118
131	33	75†	0.44	108
133	67	38	1.76	105
137	35	56†	0.63	91
142	44	36†	1.19	80*
162	57	40	1.43	97
169	31	70†	0.44	101
205	47	52†	0.90	99
220	40	28	1.43	68*

(49%) were positive for Con-A blocking factor (MG-S), 19 (10%) for toxin blocking factor and 11 (6%) appeared to have both MG-S and MG-B. A representative sample of the sera tested using the cascade is shown in Table 5.

The serum assays were used as a rough guide to determine which sera should be purified using DEAE-Sephadex or Protein-A Sepharose to yield IgG fractions containing high concentrations MG-B or MG-S. The results of the anti-receptor factor assays using purified IgG fractions confirmed the initial results using sera in 80% of the IgG fractions prepared.

The initial group of myasthenic IgG fractions was assayed by both the Con-A column method and immunoprecipitation of IgG-receptor complexes, as described by Mittag et al. (1976). The controls (AChR preincubated in either 50 or 150 µl of control IgG) showed no decrease in the ability of labelled receptors to bind to Con-A columns, no immunoprecipitation of labelled AChR, nor any interference with the toxin binding capacity of the receptor. However, preincubation of AChR with 50 µl myasthenic IgG resulted in a 14% to 41% decrease in receptor binding to Con-A columns. Further, 16% to 100% of toxin-labelled-receptors incubated with 50 µl myasthenic IgG was immunoprecipitated by anti-human IgG. Preincubation of AChR with 150 µl of myasthenic IgG resulted in a 50% inhibition of receptor binding to Con-A-Sepharose (Table 6).

Evaluation of myasthenic IgG using the Con-A/HTP cascade assay and immunoprecipitation (Table 7) revealed that in addition to inhibiting the binding of labelled AChR to Con-A-Sepharose columns, some myasthenic IgG also are capable of blocking the binding of ^{125}I-α-BuTx

Table 6

Evaluation of Myasthenic IgG Using the Con-A Assay and Immunoprecipitation

IgG	AChR Binding to Con-A-Sepharose (% Inhibition)		Immunoprecipitation (% AChR)	Apparent Anti-AChR Activity
	50 µl IgG	150 µl IgG	50 µl IgG	(nM)
M1	32	49	106	8.55
M2	35		90	7.25
M3	41	53	77	6.18
M4	35		75	5.95
M5	41		66	5.30
M6	31		37	2.07
M7	23		43	3.42
M8	18		45	3.62
M9	14		16	1.28

The values for percent inhibition of ^{125}I-α-BuTx-AChR complex binding to Con-A are relative to Con-A binding of AChR treated with control IgG = 100%. Apparent anti-AChR activity is expressed as nanomoles toxin binding sites immunoprecipitated per liter IgG.

Table 7. *Denotes toxin-blocking antibody (MG-B).

†Denotes Con-A blocking antibody (MG-S).

IgG preparations were considered positive for MG-B (*) if the total percent AChR recovered on the Con-A/HTP cascade was one standard deviation less than control, i.e., less than 88% of control. Preparations were considered positive for MG-S (+) if the increase in AChR binding to the HTP columns was greater than one standard deviation relative to control (i.e., greater than 22%).

Table 7

Evaluation of Myasthenic IgG Using the Con-A/HTP Cascade Assay and Immunoprecipitation

IgG	Con-A Column % AChR	HTP Column % AChR	Con-A/HTP Ratio	Total Recovery % AChR	Immunoprecipitation Titer (nM)
Control	88 ± 10	12 ± 10	7.33	100 ± 12	0
M90	59	12	4.9	71*	-
M94	81	20	4.05	101	-
M108	56	10	5.6	66*	-
M110	65	20	3.25	85*	-
M113	87	10	8.7	97	-
M131	55	36†	1.53	91	28.4
M137	44	46†	0.96	90	8.9
M142	44	28†	1.57	72*	-
M169	45	55†	0.82	100	3.6
M205	45	54†	0.83	99	-
M250	60	10	6.0	70*	-

to AChR. Furthermore, immunoprecipitation titer did not necessarily correlate with the levels of anti-receptor factor which blocked toxin binding to receptors or the anti-receptor factor which blocked receptor binding to Con-A-Sepharose. Similar findings were reported for AChR treated with myasthenic sera (Mittag et al., 1976).

8. Effects of myasthenic IgG containing factor-I on the binding of AChR to the Con-A/HTP cascades HTP columns and immunoprecipitation

Myasthenic IgG containing factor-I (MG-I) does not interfere with the binding of ^{125}I-α-BuTx to AChR as determined by either the Con-A/HTP cascade and HTP assay methods. MG-I had no effects on the ratio of Con-A to HTP binding in the Con-A/HTP cascade assay relative to both normal IgG and normal saline, nor did it change the total binding capacity of toxin for the receptor, as measured by the HTP column method. However, MG-I does bind to AChR in a concentration dependent manner as evidenced by anti-human IgG immunoprecipitation of labelled AChR that had been preincubated with MG-I. A maximal precipitation of 103 ± 2.5% of the labelled receptors was obtained. The ED_{50} was 0.94 ± 0.06 μM and the Hill coefficient was 1.1 ± 0.07 (Fig. 12). The same results were obtained when AChR were labelled prior to incubation with MG-I.

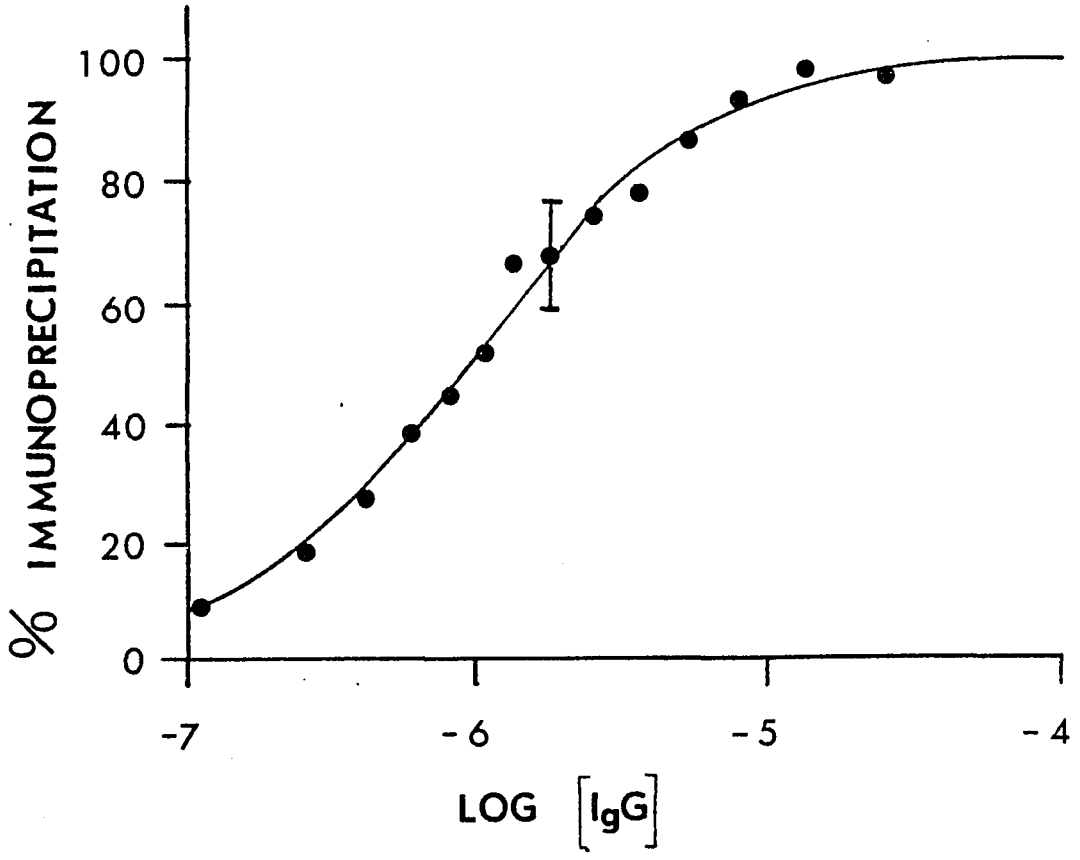
9. Effects of myasthenic IgG containing factor-I on the kinetics of ^{125}I-α-BuTx binding to AChR

Preincubation of AChR with MG-I does not change the kinetics of ^{125}I-α-BuTx binding to receptors in any manner when compared to the kinetics of toxin binding to receptors preincubated in control IgG or normal saline (Table 3). In each case, the two component model pro-

Figure 12. AChR were incubated with MG-I for 24 hours at $4^{o}C$ and then labelled with ^{125}I-α-BuTx for 24 hours. Samples were divided for assay by the HTP column method or by immunoprecipitation with anti-human IgG. No inhibition of toxin binding was observed at any concentration of MG-I tested (data not shown). Data illustrated are the average for three experiments using three different AChR preparations. Bar represents average standard deviation of the data points. Maximum percent AChR precipitated was 103 ± 2.5% (ED_{50} = 0.94 ± 0.06 μM, Hill coefficient = 1.1 ± 0.07).

Figure 12

Immunoprecipitation of AChR Treated with MG-I



vided a better description of the data than did the one component model. The rate constant for the first component, k_1, is 8.22 ± 0.7 x $10^4 M^{-1}s^{-1}$ and 0.35 ± 0.04 x $10^4 M^{-1}s^{-1}$ for k_2 for the control IgG treated receptors, while the values for the MG-I treated receptors are 8.26 ± 0.91 x $10^4 M^{-1}s^{-1}$ and 0.30 ± 0.03 x $10^4 M^{-1}s^{-1}$ for k_1 and k_2, respectively (Fig. 13); values which are not significantly different from the control values ($P > .05$).

10. Effects of myasthenic IgG containing factor-S on the binding of AChR to the Con-A and Con-A/HTP cascade columns

The initial investigation of the interaction of AChR with myasthenic IgG containing factor-S (MG-S) was performed using the Con-A column assay. MG-S (M1-M5, Table 6) caused a dose-dependent inhibition of labelled receptor binding to Con-A-Sepharose affinity columns (Fig. 14). The maximum inhibition was found to be 54.8 ± 4.4%, with an ED_{50} of 6.67 ± 0.85 μM and Hill coefficient of 1.09 ± 0.14. This inhibition could result from either an inhibition of toxin binding to the receptor or inhibition of receptor binding to Con-A-Sepharose. Substitution of the Con-A/HTP cascade assay for the Con-A column method revealed that the action of MG-S (M131, M137, M169 or M205, Table 7) is to alter the ratio of labelled AChR binding between the Con-A and HTP columns without interfering with the total toxin binding capacity of the receptor. (Table 7. See also next section.) This confirmed the results found comparing the Con-A assay method with immunoprecipitation (Mittag et al., 1978).

11. Effects of prelabelling AChR prior to exposure to myasthenic IgG containing factor-S

Detailed dose-response curves were established for three myas-

Figure 13. The two component model provided a significantly better description of the data for toxin binding to both saline and MG-I treated AChR. In this typical experiment k_1 = $8.7 \times 10^4 M^{-1}s^{-1}$ and $k_2 = 0.34 \times 10^4 M^{-1}s^{-1}$ for control IgG treated AChR (closed triangles) while $k_1 = 8.0 \times 10^4 M^{-1}s^{-1}$ and $k_2 = 0.29 \times 10^4 M^{-1}s^{-1}$ for MG-I treated AChR (open triangles). Similar results were obtained in two other experiments using two different receptor preparations (see Table 3). The differences between the respective rate constants was found to be not significant using the two tailed Student's t-test ($P > .05$).

Figure 13

Kinetics of ^{125}I-α-BuTx Binding to MG-I or Control IgG Treated AChR

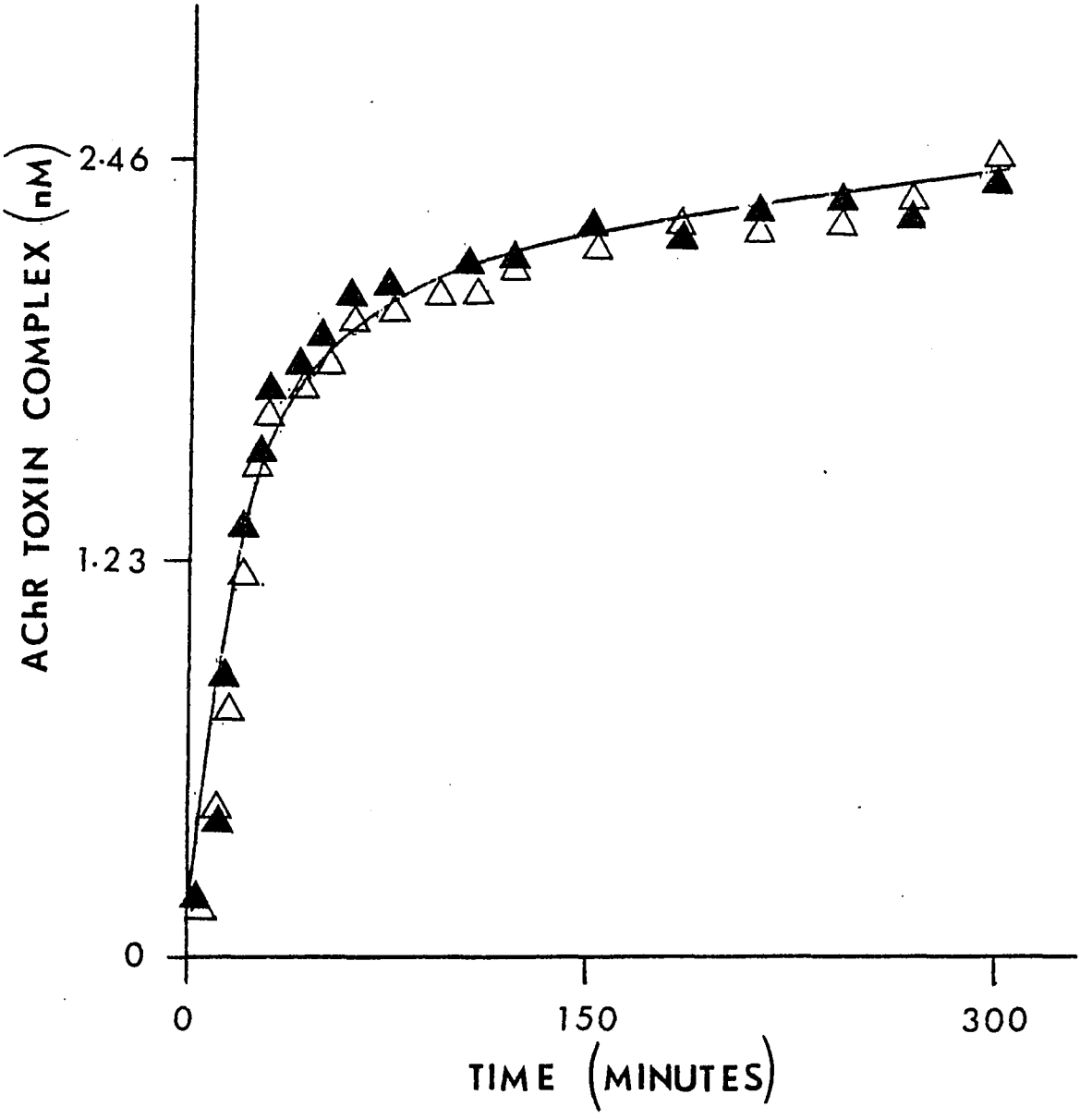
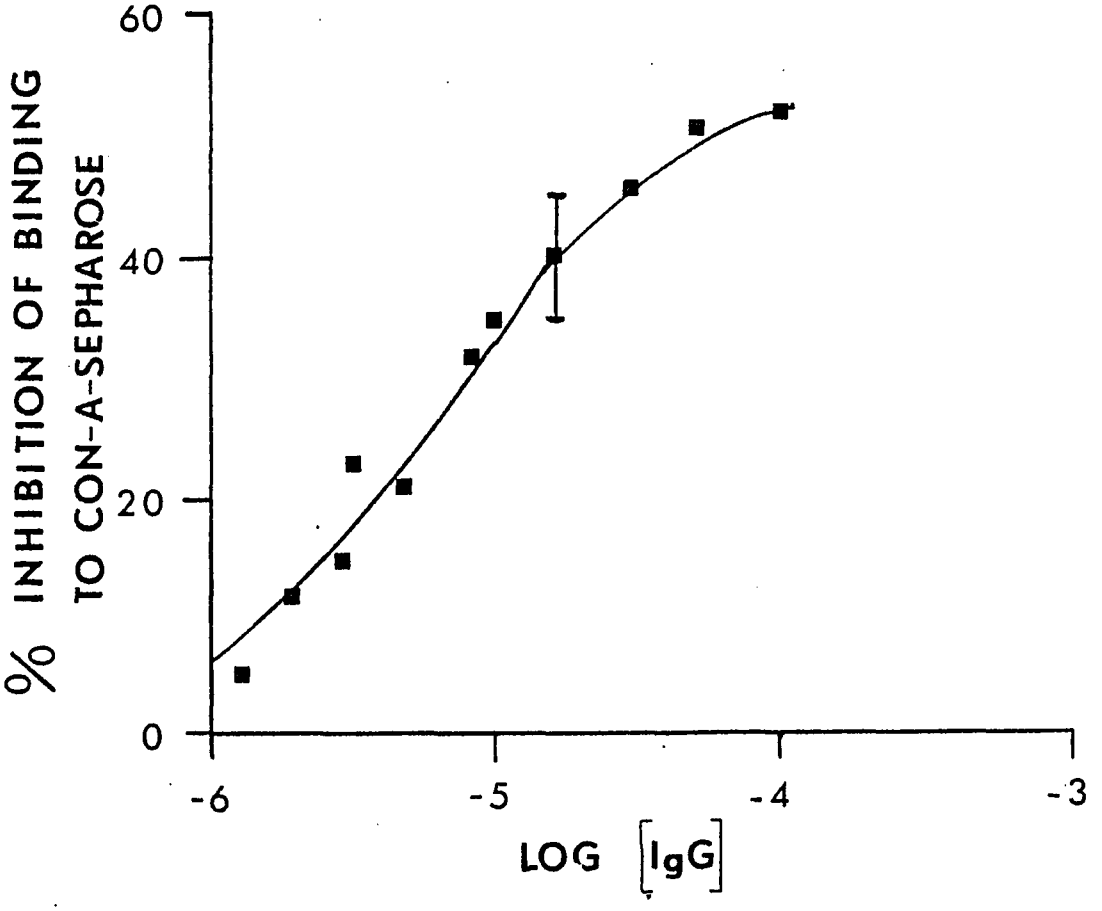


Figure 14. AChR treated with MG-S prior to labelling with ^{125}I-α-BuTx were assayed using the Con-A column method. Data are the average of five experiments using three different receptor preparations and four different MG-S IgG preparations. The Bar represents the average standard deviation of the data points. The maximum percent inhibition of AChR binding to Con-A-Sepharose was 54.8 ± 4.4% (ED_{50} = 6.67 ± 0.84 μM, Hill coefficient = 1.09 ± 0.14).

Figure 14

Inhibition of AChR Binding to Con-A-Sepharose

Following Treatment with MG-S



thenic IgG preparations containing factor-S (131, 169, 205), all of which were previously determined to have approximately equivalent ability to inhibit the binding of labelled receptors to Con-A columns without altering the total binding capacity of the receptor for toxin (Table 7). In this set of experiments, the Con-A/HTP cascade was used.

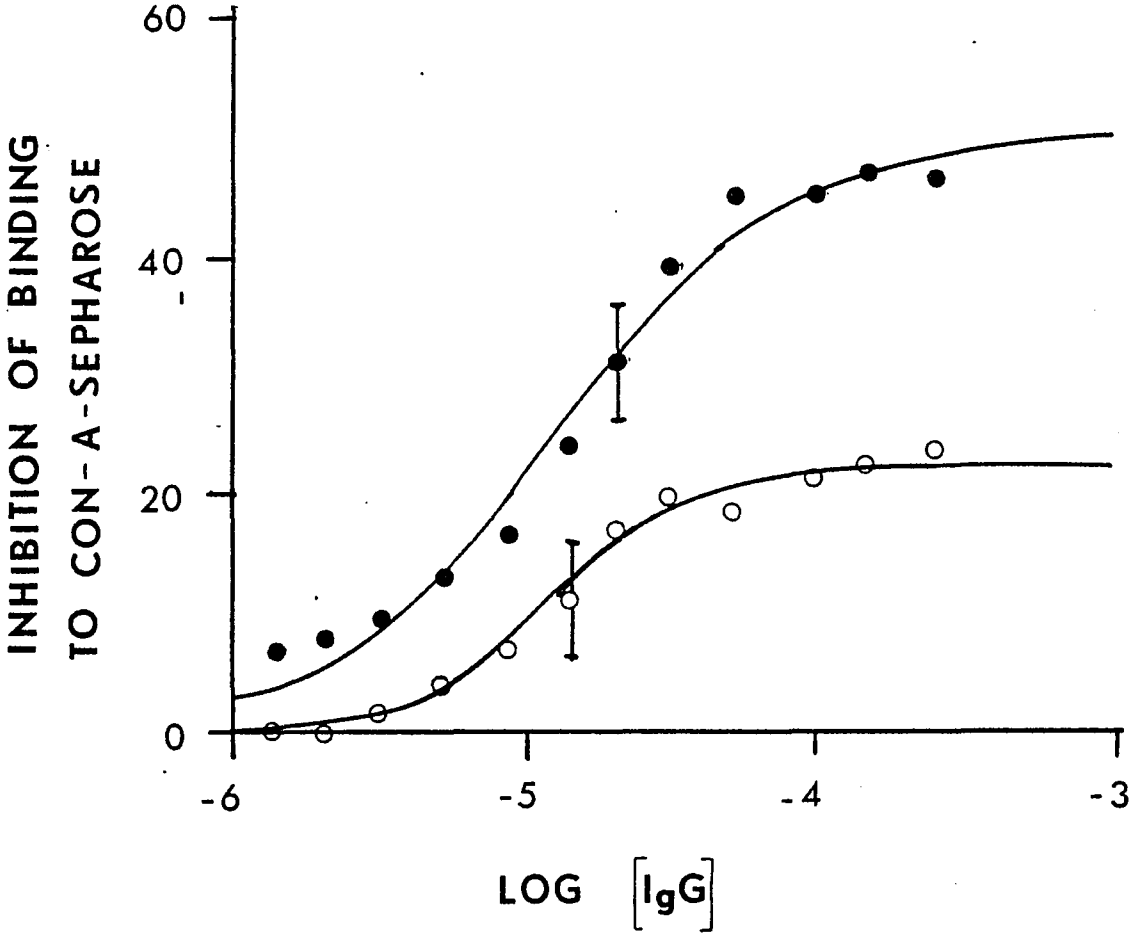
The inhibition of binding to Con-A-Sepharose of receptors preincubated with MG-S prior to labelling was almost identical to the inhibition obtained using the earlier MG-S preparations (Fig. 15). The maximal inhibition observed was 50.5 ± 2.5%, with an ED_{50} of 13.4 ± 2.0 μM and Hill coefficient of 1.1 ± 0.14. The sum of the labelled receptor on the Con-A and HTP columns of the cascade at each concentration of MG-S tested was equal to that found for control IgG and normal saline, showing that although MG-S caused a dose-dependent inhibition of labelled receptor binding to Con-A, it did not alter the total binding capacity of the receptor for toxin. This is in agreement with the results obtained using the Con-A method in parallel with immunoprecipitation.

Receptors labelled with ^{125}I-α-BuTx prior to exposure to MG-S also showed a dose-dependent inhibition of binding to Con-A-Sepharose. MG-S did not cause dissociation of toxin-receptor complexes. However, the maximum inhibition of Con-A binding observed for the prelabelled receptors was 22.8 ± 0.7% (ED_{50} of 13.3 ± 1 μM, Hill coefficient of 1.26 ± 0.22). This represents approximately half the inhibition observed when receptors are preincubated with MG-S prior to labelling with ^{125}I-α-BuTx (Fig. 15).

Figure 15. AChR exposed to MG-S prior to (closed circles) or after (open circles) labelling with ^{125}I-α-BuTx were assayed using the Con-A/HTP cascade method. In both cases AChR inhibited from binding to Con-A-Sepharose were recovered on the HTP column of the cascade. The data points are the average of three experiments using three different AChR preparations and three different MG-S IgG preparations. The maximum percent inhibition for AChR labelled after exposure to MG-S was 50.5 ± 2.5% (ED_{50} = 13.4 ± 2 μM, Hill coefficient = 1.1 ± 0.14) and 22.8 ± 0.7% (ED_{50} = 13.3 ± 1 μM, Hill coefficient = 1.26 ± 0.22) for AChR labelled prior to exposure to MG-S.

Figure 15

Inhibition of AChR Binding to Con-A-Sepharose: Effect of Labelling Receptors Before or After Incubation with MG-S



12. Effects of preincubation with myasthenic IgG containing factor-S on the kinetics of toxin binding to AChR

As noted earlier (Table 3), the two component model provides a significantly better fit to the data for the kinetics of toxin binding to AChR preincubated with control IgG. The values for the two rate constants were 8.22 ± 0.7 x $10^4 M^{-1}s^{-1}$ and 0.35 ± 0.04 x $10^4 M^{-1}s^{-1}$ for k_1 and k_2, respectively. A typical experiment is shown in Figure 4.

The two component system also was found to fit the data for the kinetics of toxin binding to receptors pretreated with MG-S for receptors binding to both the Con-A and HTP columns of the cascade (Table 3 and Fig. 16). However, the rate constants (k_1 = 6.4 ± 0.97 x $10^4 M^{-1}s^{-1}$ and k_2 = 0.11 ± 0.01 x $10^4 M^{-1}s^{-1}$) were significantly different than those found for the control ($P < 0.05$). The concentration of complex found after 24 hours under saturating toxin concentrations at 4°C was 3.19 nM for the receptors treated with control IgG and 2.95 nM for MG-S pretreated receptors, values which are not significantly different. Thus, the data show that MG-S inhibits the binding of labelled receptors to Con-A-Sepharose columns, and decreases the rate of toxin binding to the receptors but does not alter the total toxin binding capacity of the receptor.

13. Effects of Protein-A purified myasthenic IgG containing factor-S on the binding of AChR to Con-A-Sepharose

Protein-A purified IgG fractions of patients M205 and M218 were found to induce the same percent inhibition of receptor binding to Con-A-Sepharose columns as the DEAE prepared IgG fractions of the same patients (Table 8) when assayed at 50 μM IgG. In each case re-

Table 8

AChR Binding to Con-A-Sepharose: Effect of Preincubation with Myasthenic IgG Fractions Containing MG-S Purified by DEAE-Sephadex or Protein-A-Sepharose Chromatography

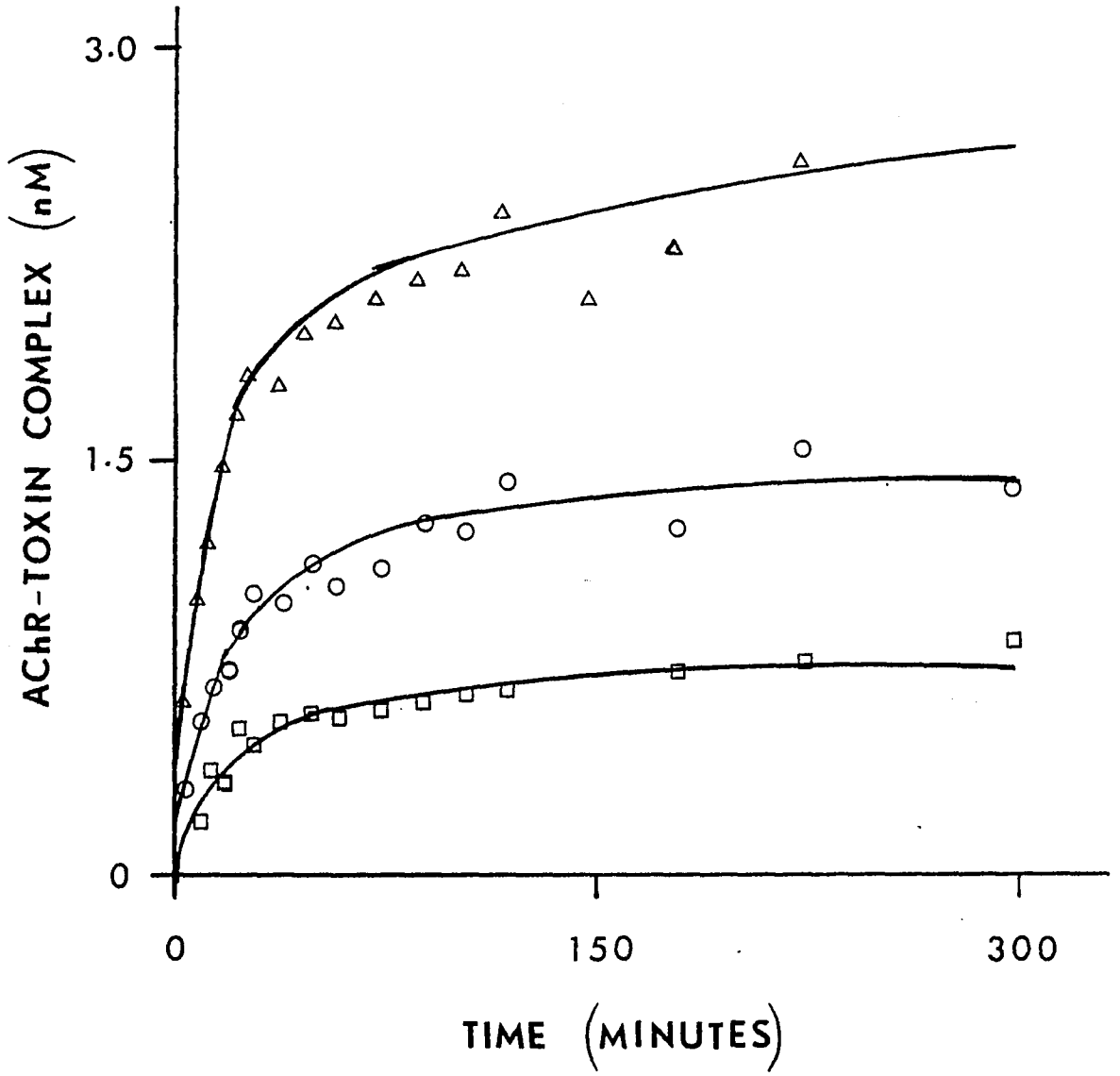
IgG Source/Method of Purification	Con-A-Sepharose % AChR	Hydroxylapatite % AChR	Total Recovery % AChR
Control/DEAE	89	11	100
Control/Protein-A	87	13	100
M205/DEAE	45	54	99
M205/Protein-A	50	45	95
M218/DEAE	49	47	96
M218/Protein-A	47	58	105

Values are expressed as percent of the total AChR recovered following treatment with control IgG as measured by the Con-A/HTP cascade assay method. These data indicate that the DEAE and Protein-A purification of IgG from myasthenic sera produce equivalent IgG preparations containing the myasthenic anti-receptor antibody MG-S.

Figure 16. AChR were treated with control or MG-S IgG for 24 hours prior to the addition of ^{125}I-α-BuTx. Samples taken from the reaction mixture at various times were assayed using the Con-A/HTP cascade. Computer analysis of the data indicates that the two component model provides a significantly better fit to the data for toxin binding to control IgG treated AChR binding to Con-A (triangles) as well as MG-S treated AChR binding to Con-A (circles) and HTP (squares). In this typical experiment, the rate constants for control IgG treated AChR were 8.2 x $10^4 M^{-1}s^{-1}$ and 0.37 x $10^4 M^{-1}s^{-1}$. MG-S treated AChR binding to Con-A and HTP were found to have corresponding rate constants of 6.2 x $10^4 M^{-1}s^{-1}$ and 0.12 x $10^4 M^{-1}s^{-1}$. No inhibition of toxin binding ocurred while 45.5% inhibition of AChR binding to Con-A-Sepharose was found for MG-S treated AChR. Similar results were obtained in two other experiments using two different AChR preparations and two different MG-S IgG preparations (see Table 3).

Figure 16

Kinetics of ^{125}I-α-BuTx Binding to MG-S Treated AChR



ceptors which did not bind to the Con-A columns were recovered on the HTP columns of the cascade. For both sera factor-S activity was eluted from the Protein-A columns. No Factor-B activity was observed in either preparation.

14. Effects of myasthenic IgG containing factor-B on the binding of ^{125}I-α-BuTx to AChR

Myasthenic IgG containing factor-B (MG-B) caused a dose-dependent inhibition of ^{125}I-α-BuTx binding to AChR preincubated with MG-B prior to labelling (Fig. 17). The maximal inhibition obtained was 30.23 ± 1.85% (ED_{50} = 5.76 ± 0.43 μM, Hill coefficient = 1.95 ± 0.23), as measured by the HTP assay method. No inhibition of toxin binding was observed when receptors were labelled with toxin prior to incubation with MG-B.

15. Apparent equilibrium binding of ^{125}I-α-BuTx to AChR: effects of preincubation of AChR with myasthenic IgG containing factor-B

As described above, the equilibrium binding of toxin to receptor pretreated with saline or control IgG results in an apparent K_D of 0.72 ± 0.18 nM (Fig. 6) and the Scatchard analysis of the data results in a straight line, indicating the presence of a homogeneous population of receptors (Fig. 7).

The apparent K_D obtained when receptors were preincubated for 24 hours with 50 μM MG-B prior to labelling was found to be 0.4 ± 0.08 nM (Fig. 18). The difference in apparent K_D was found to be significantly different than the control ($p < 0.01$). Toxin binding to MG-B treated receptors was decreased by 31.3 ± 0.5% relative to control. Scatchard analysis of the data indicates the presence of a homogeneous population of receptors (Fig. 19).

Figure 17. AChR pretreated with MG-B IgG for 24 hours were labelled for 24 hours at $4^{o}C$ and assayed using the HTP column method. Data are the average for five experiments using three different MG-B IgG preparations and four AChR preparations. Bar represents average standard deviation of data points. The maximum inhibition of toxin binding was 30.23 ± 1.85% (ED_{50} = 5.76 ± 0.43 μM, Hill coefficient = 1.95 ± 0.23).

Figure 17

Inhibition of ^{125}I-α-BuTx Binding to MG-B Treated AChR

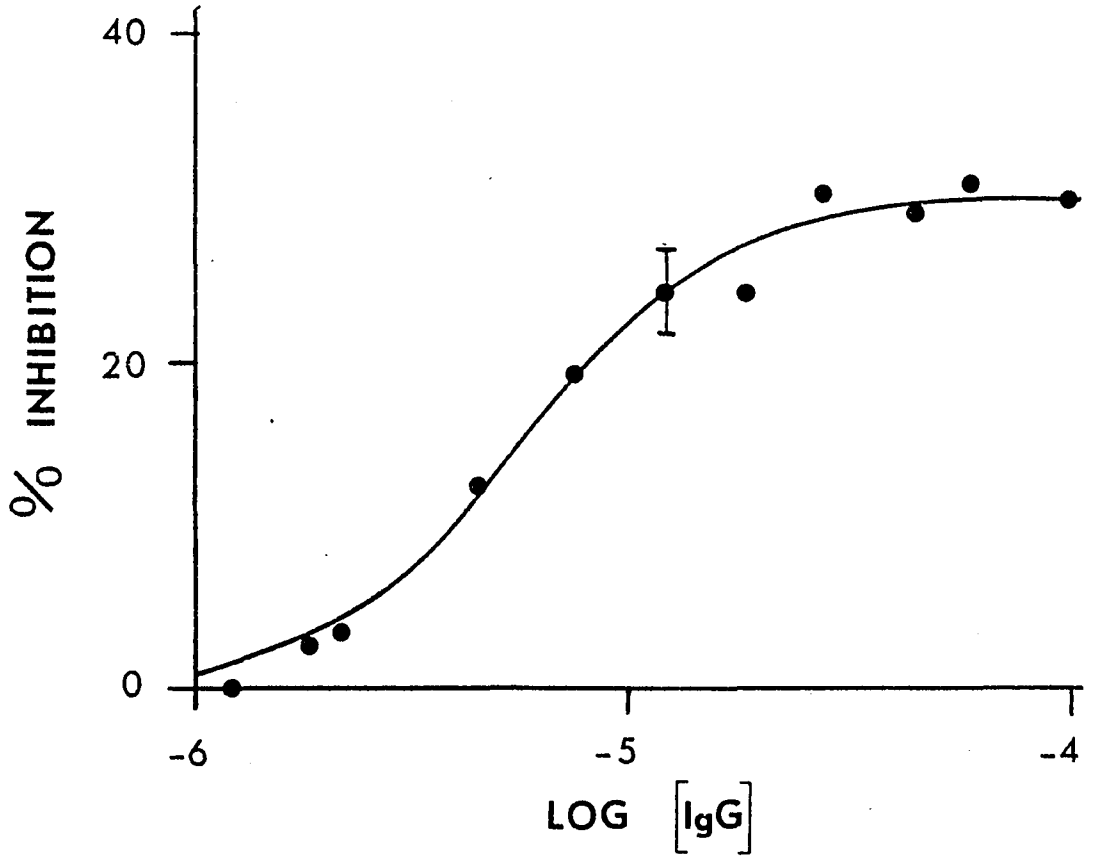


Figure 18. AChR were treated with control or MG-B IgG for 24 hours and labelled with varying concentrations of ^{125}I-α-BuTx for another 24 hours at 4°C. All samples were assayed using the HTP column method. K_{Dapp} in this typical experiment was 0.4 nM for MG-B treated AChR (closed diamonds) and 0.6 nM for control IgG treated AChR (open diamonds). In addition, 31.5% inhibition of toxin binding to MG-B treated AChR was observed. Similar results were obtained in two other experiments using two different AChR preparations and two different MG-B IgG preparations.

Figure 18

Apparent Equilibrium Binding of

^{125}I-α-BuTx to MG-B Treated AChR

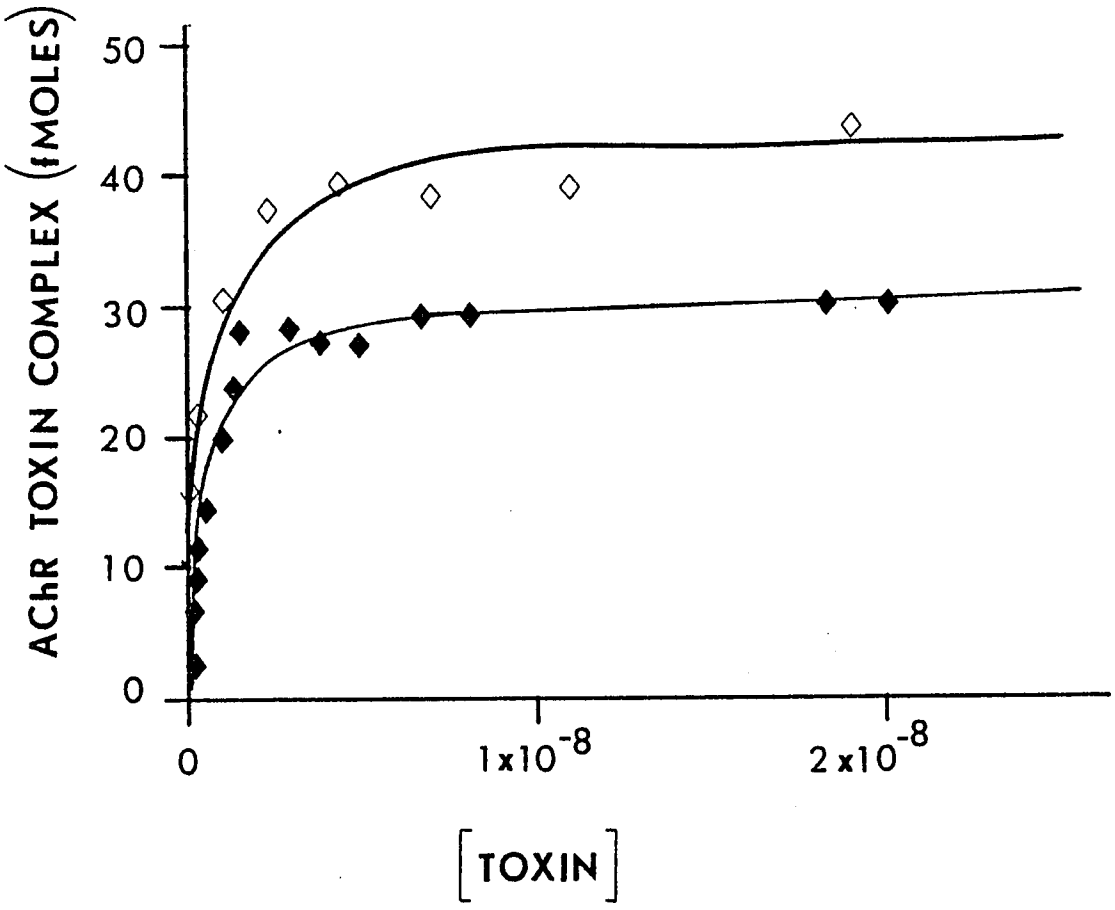
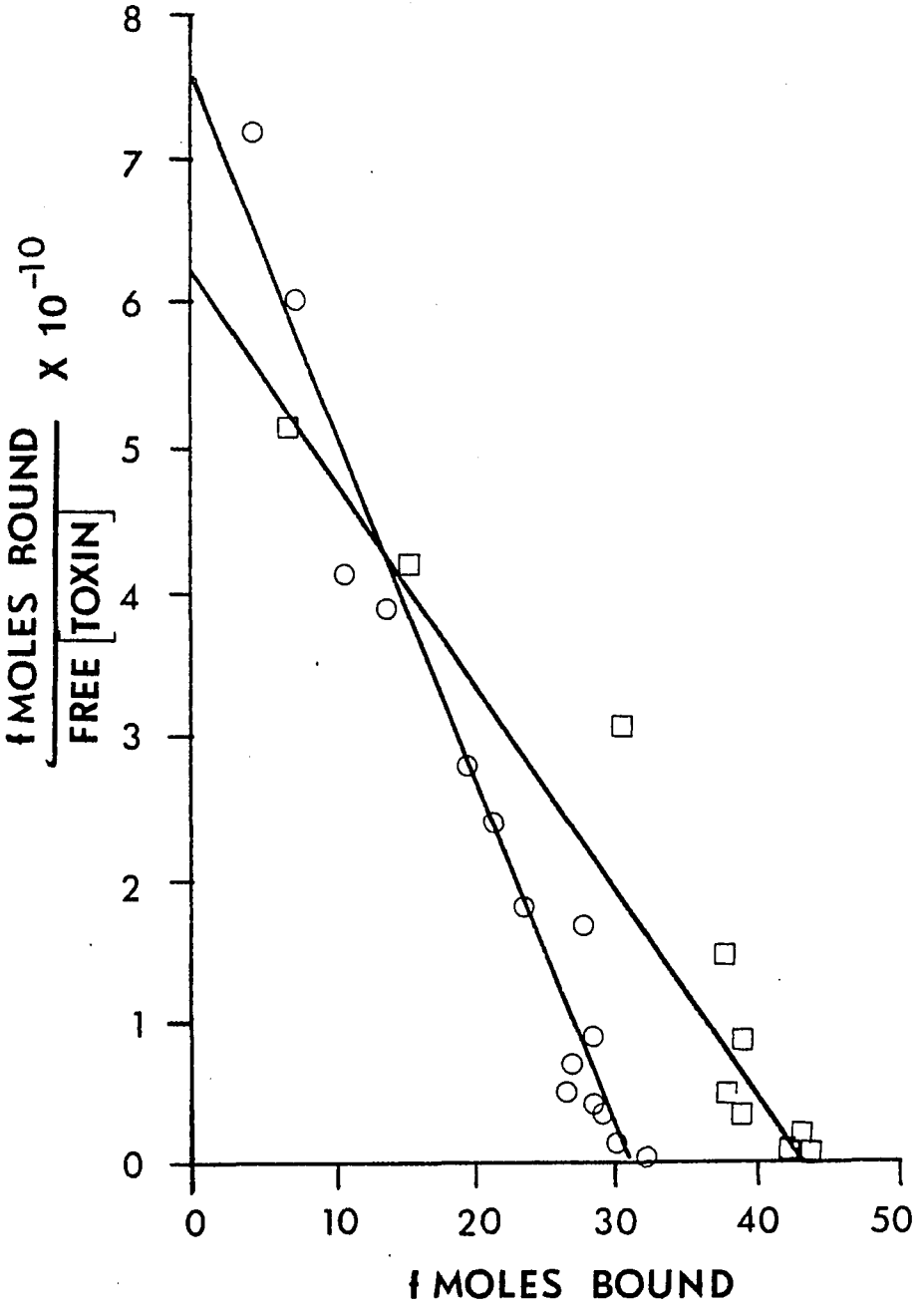


Figure 19. Scatchard analysis of the data shown in Fig. 18 indicates the presence of a homogeneous population of AChR for both control (squares) and MG-B (circles) treated AChR. The mean K_{Dapp} for MG-B IgG treated AChR (0.4 ± 0.08 nM) was found to be significantly different ($P < .01$, using the two-tailed Student's t-test) than the mean K_{Dapp} for control AChR (0.72 ± 0.18 nM).

Figure 19

Equilibrium Binding of Toxin to MG-B Treated AChR



16. Effects of Protein-A purified myasthenic IgG containing factor-B on the binding of ^{125}I-α-BuTx to AChR

Protein-A purified IgG fractions were prepared from the sera of three patients (M250, M108, M174) previously shown to have factor-B. The Protein-A purified IgG fractions from two of these three patients, M250, M108, exhibited the same percent maximum inhibition of toxin binding as the DEAE purified IgG fractions when tested at 50 μM (Table 9). Purification of DEAE prepared IgG and the serum of patient M250 using Protein-A-Sepharose affinity chromatography resulted in fractions of equal IgG content and equivalent inhibitory activity. This indicates that the Protein-A and DEAE purified IgG preparations are equivalent.

All of the factor-B activity found in these three preparations was associated with the first acetate fraction eluted from the Protein-A column. No activity was found in the phosphate fractions nor any of the other acetate fractions.

The third Protein-A preparation (M174) resulted in an IgG fraction capable of inhibiting 100% of the receptors from binding toxin when tested at 50 μM IgG (Table 9). A detailed dose-response curve showed that the ED_{50} (7.8 ± 0.6 μM) and Hill coefficient (1.9 ± 0.24) for the inhibition of toxin binding were the same as those found for the DEAE purified IgG fractions (Fig. 20). The percent maximum inhibition using MG-B-174 was 103 ± 4.7%, in contrast to 30.2 ± 1.85% for the DEAE purified IgG fractions from M90, M108 and M250, as well as Protein-A purified M250. The inhibitory activity was associated with the first and second acetate fractions eluted from the Protein-A column. No inhibition of toxin binding was observed with any of the

Table 9

Inhibition of Toxin Binding to AChR: Comparison of Effects of Pretreatment with MG-B Purified by DEAE-Sephadex or Protein-A-Sepharose Chromatography

MG-B Source	Purification Method	% Maximum Inhibition of Toxin Binding to AChR
M250 serum	DEAE	29.0 ± 4.1
M250 serum	Protein-A	31.5 ± 3.2
M250 DEAE IgG	Protein-A	30.0 ± 1.9
M108	DEAE	32.0 ± 2.4
M108	Protein-A	29.0 ± 5.0
M174	Protein-A	103.0 ± 7.1

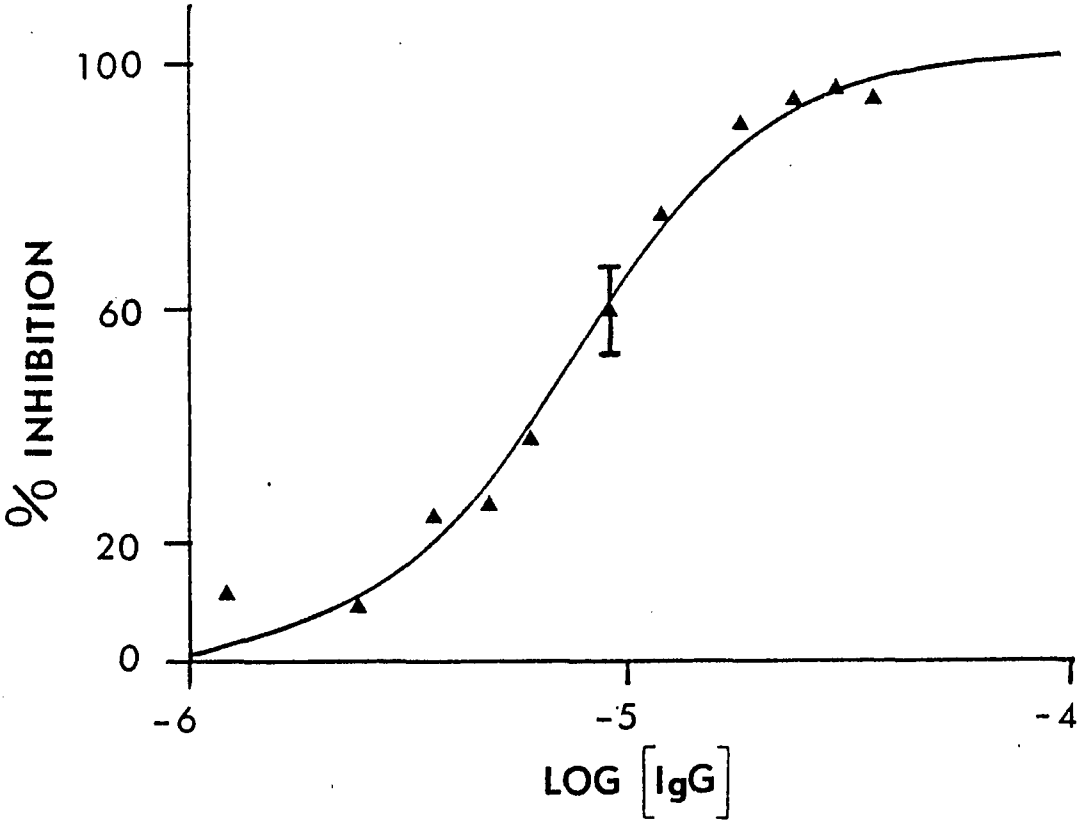
All IgG preparations were tested at 50 μM IgG using AChR labelled after exposure to MG-B. All samples were assayed using the HTP column method. The values are expressed as percent maximum inhibition of toxin binding to AChR with respect to binding of toxin to AChR treated with control IgG, and are the average (± S.D.) of three experiments.

Figure 20. AChR were incubated with varying concnetrations Protein-A-Sepharose purified MG-B-174 for 24 hours and labelled with ^{125}I-α-BuTx for another 24 hours. All samples were assayed using the HTP column method. The maximum percent inhibition of toxin binding was 103 ± 4.7% (ED_{50} = 7.8 ± 0.6 μM, Hill coefficient = 1.90 ± 0.24). Data are the average of three experiments using two different receptor preparations.

Figure 20

Effects of Protein-A-Sepharose Purified

MG-B-174 on the Binding of ^{125}I-α-BuTx to AChR



above IgG preparations when the receptors were labelled with toxin prior to incubation with IgG. At each concentration of M174 Protein-A purified IgG tested, it was not possible to immunoprecipitate labelled receptors regardless of whether the AChR were incubated with MG-B before or after labelling with toxin. Thus, the Protein-A purified IgG fraction of M174, unlike M250 and M108, contains no MG-I. Furthermore, when assayed on the Con-A/HTP cascade, no increase in the percent of AChR binding to the HTP columns, relative to control, was noted at any concentration of MG-B-174 tested. This strongly suggests that no factor-S is present in the Protein-A purified IgG fraction of M174. Therefore, the serum of patient M174 is unique in that it contains only MG-B.

17. Binding of Lens culinaris purified AChR to the Con-A/HTP cascade: effects of incubation with MG-B or MG-S

Lens culinaris purified AChR exhibit different sensitivity to incubation with MG-B and MG-S than has been observed for hydroxylapatite purified AChR (Table 10). No inhibition of toxin binding is observed when LCA AChR are incubated with MG-B prior to labelling. The concentration of MG-B used was 10 times greater than the ED_{50} found for inhibition of toxin binding to hydroxylapatite purified receptors.

MG-S treated LCA purified receptors showed a 50% maximum inhibition of toxin-receptor antibody complex binding to Con-A-Sepharose, with complete recovery of the displaced complexes on the HTP column of the cascade. The 50% maximum inhibition was observed for receptors exposed to MG-S prior to or after labelling. Earlier experiments using HTP prepared AChR showed that MG-S induced a 54.8 $\pm$ 4.4% and

Table 10

Effects of MG-S and MG-B on AChR Prepared Using HTP or LCA Chromatography: Comparison of Pre versus Postlabelled AChR

Receptor Preparation/Labelling	MG-S % Inhibition of AChR Binding to Con-A-Sepharose	MG-B % Inhibition of α-BuTx Binding to AChR
HTP/Prelabelled	22.8 ± 0.7	0
HTP/Postlabelled	54.8 ± 4.4	30.2 ± 1.9
LCA/Prelabelled	46.0 ± 2.9	0
LCA/Postlabelled	49.0 ± 5.7	0

HTP or LCA prepared AChR were labelled prior to or after exposure to 44 μM MG-S or MG-B. AChR treated with MG-S were assayed using the Con-A/HTP cascade. All MG-S treated samples exhibited 100% recovery of AChR inhibited from binding to Con-A-Sepharose on the HTP column of the cascade. Receptors treated with MG-B were assayed using the HTP column method. All values shown are relative to those obtained for AChR treated with control IgG, and are the average (± S.D.) for four experiments.

22.8 ± 0.7% inhibition of complex binding to Con-A-Sepharose for post- and prelabelled AChR, respectively.

18. Kinetics of ^{125}I-α-BuTx binding to LCA purified AChR: effects of preincubation with MG-S

As noted previously, the binding of toxin to LCA purified AChR can be described by a single rate constant, 11.8 ± 1.0 x $10^4 M^{-1}s^{-1}$ (Table 3, Fig. 9). The association rate constant obtained for Lens purified receptors pretreated with MG-S, for receptors binding to Con-A-Sepharose and HTP, was 8.0 ± 0.4 x $10^4 M^{-1}s^{-1}$ (Fig. 21). This value is significantly smaller than that obtained for Lens AChR pretreated in saline or control IgG ($P < .05$). Complex binding to Con-A-Sepharose was inhibited by 52.2%. This is similar to MG-S pretreatment of hydroxylapatite AChR, in that the rate of the toxin binding for each site was slowed by MG-S and Con-A-Sepharose binding was inhibited by approximately 50%. The obvious difference is the absence of the slow toxin binding site.

19. Interaction of Concanavalin-A and myasthenic IgG containing factor-I with labelled AChR

Preincubation of labelled receptors with Con-A prior to incubation with MG-I, or MG-I prior to Con-A, results in immunoprecipitation of 100% of the receptors when either anti-Con-A antiserum or anti-human IgG are added (Table 11). These results indicate that the binding sites for Con-A and MG-I on the AChR are independent and separate entities, and also are different from the toxin-binding sites.

An interesting observation was made in these experiments. As expected, no immunoprecipitation occurred with anti-human IgG when receptors were treated with either Con-A or control IgG. It was noted,

Figure 21. LCA purified AChR were treated with MG-S IgG for 24 hours prior to labelling with ^{125}I-α-BuTx. Samples were removed from the reaction mixture at various times and assayed using the Con-A/HTP cascade method. AChR treated with control IgG bound solely to the Con-A column. Toxin binding to these receptors as well as those treated with MG-S and binding to Con-A or HTP, is best described by a one component model. In this typical experiment, the rate constant for the control IgG treated AChR was 12.1 x $10^4 M^{-1}s^{-1}$ (diamonds), while the rate constant for MG-S treated AChR binding to Con-A (triangles) and HTP (circles) was 7.8 x $10^4 M^{-1}s^{-1}$. Similar results were obtained in two other experiments using two different MG-S IgG preparations and two different LCA purified AChR preparations.

Figure 21

Kinetics of ^{125}I-α-BuTx Binding to LCA

Purified AChR Treated with MG-S

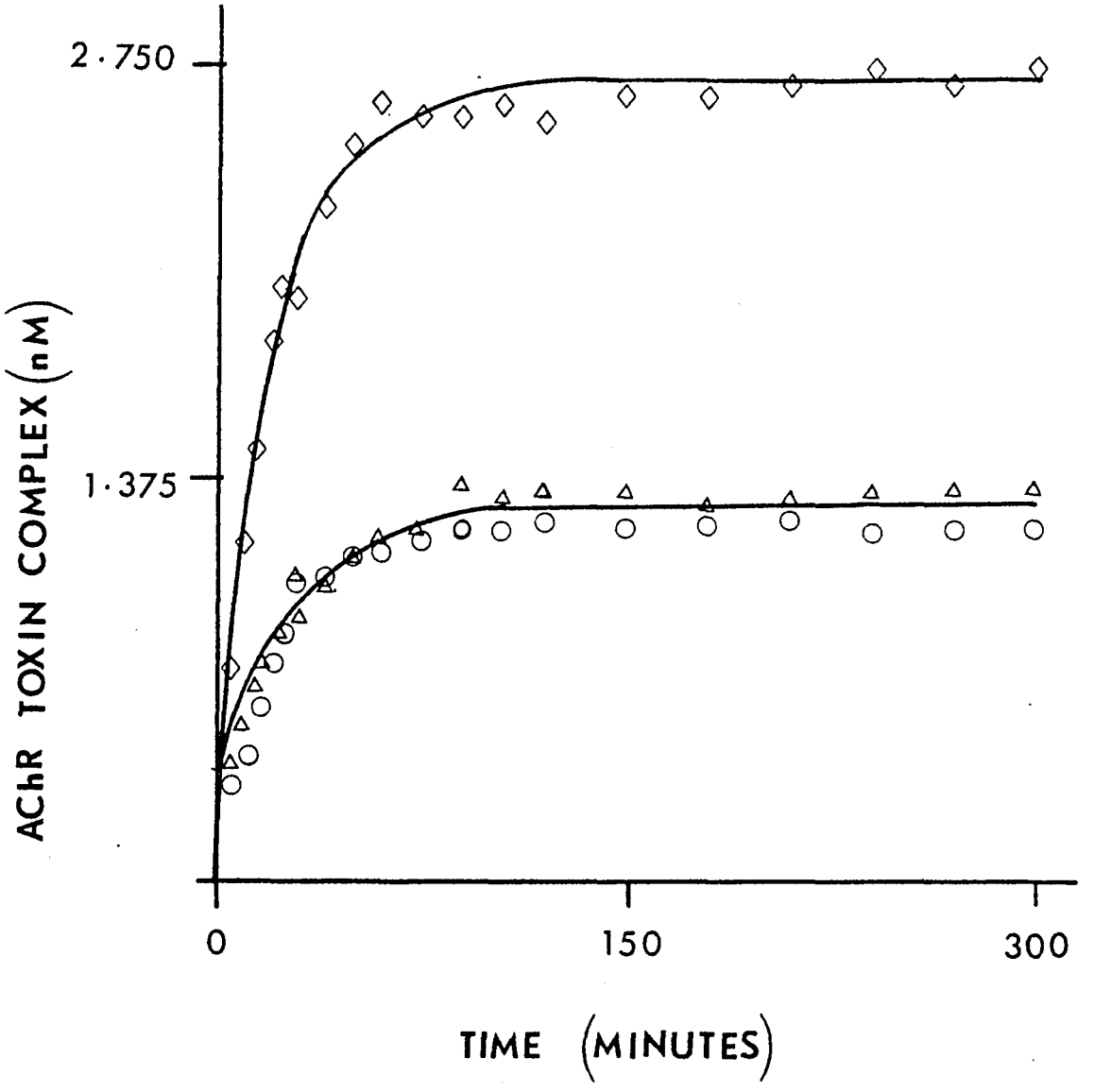


Table 11. Samples incubated in presence or absence of 10 mM α-methyl-mannoside prior to various treatment schedules were divided for assay on HTP columns, and immunoprecipitation by anti-human IgG or anti-Con-A antisera. The values shown are the average (± S.D.) for three experiments using three different receptor preparations and are relative to the total AChR-toxin complex found using the HTP assay for AChR treated with saline or control IgG.

Table 11

Interaction of Con-A and MG-I with ^{125}I-α-BuTx Labelled AChR:

Comparison of Immunoprecipitation by Anti-Con-A or Anti-Human IgG

Treatment	% Immunoprecipitation			
	Anti-Human IgG		Anti-Con-A	
	mannoside (10mM)	no mannoside	mannoside (10mM)	no mannoside
Saline	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Con-A	2.3 ± 2.0	6.5 ± 1.5	94.0 ± 14.0	113.0 ± 3.0
Normal IgG	0 ± 0	0 ± 0	1.7 ± 2.4	0 ± 0
MG-I	111.0 ± 9.4	117.5 ± 2.5	1.3 ± 1.9	0 ± 0
Con-A/Normal IgG	9.3 ± 7.0	107.0 ± 3.0	104.0 ± 8.8	103.5 ± 2.5
Con-A/MG-I	97.0 ± 5.9	99.5 ± 18.0	95.7 ± 13.1	104.0 ± 1.0
Normal IgG/Con-A	5.3 ± 5.0	98.0 ± 8.0	86.0 ± 12.0	103.5 ± 5.5
MG-I/Con-A	94.2 ± 3.1	107.0 ± 9.0	93.0 ± 7.0	104.0 ± 6.0

however, that receptors treated with Con-A followed by control IgG, or vice versa, were completely immunoprecipitated with anti-human IgG. This could result only from a cross-linking by Con-A between the receptors and control IgG since the control IgG contained no antibodies which recognized AChR. This effect was abolished by adding 10 mM α-methyl-D-mannoside to the incubation prior to the addition of either Con-A or IgG. This concentration of mannoside was sufficient to interfere with the Con-A IgG interaction, thus preventing the cross-linking. It did not alter the Con-A receptor interaction, since 100% of the AChR-Con-A complexes were precipitated by anti-Con-A in the presence of mannoside. Inhibition of Con-A binding to AChR requires at least 150 mM mannoside (Brockes and Hall, 1975b). The Con-A-IgG interaction had no effect on the HTP assay nor immunoprecipitation with anti-Con-A antiserum.

20. Interaction of Concanavalin-A and myasthenic IgG containing factor-B with AChR: effects on the binding of ^{125}I-α-BuTx to AChR

Preincubation of AChR with 15 μM Con-A or 50 μM MG-S separately results in a 39.5 ± 3.4% and 28 ± 3.5% inhibition of toxin binding to AChR respectively. These values compare well with the values found for Con-A and MG-B induced inhibition of toxin binding (Table 12). However, Con-A and MG-B together produce approximately a 70% inhibition of toxin binding, regardless of the order of incubation. No inhibition was observed when prelabelled AChR were treated similarly. These results indicate that the effects of Con-A and MG-B are mediated through separate and independent binding sites and are additive.

Table 12

Interaction of Con-A and MG-B with AChR:

Effects on Binding of ^{125}I-α-BuTx to AChR

Treatment	% Inhibition of Toxin Binding to AChR
Saline	0
Control IgG	0
Con-A	39.5 ± 3.4
MG-B	28.0 ± 3.5
Con-A followed by Control IgG	42.0 ± 5.1
Con-A followed by MG-B	71.0 ± 4.8
Control IgG followed by Con-A	37.0 ± 2.8
MG-B followed by Con-A	69.0 ± 7.2

The values are the average (± S.D.) for three experiments with three different AChR preparations. All samples were assayed using the HTP column method.

V. DISCUSSION

The glycoprotein nature of the nicotinic AChR has been well established by several investigators (Meunier et al., 1974; Mattson and Heilbronn, 1975; Vandlen et al., 1976; Brockes and Hall, 1975b). The ability of Con-A to bind to the receptor, indicating the presence of α-D-mannoside and α-D-glucoside residues (Sharon and Lis, 1972), has been exploited to purify the receptor by means of affinity chromatography (Almon and Appel, 1976; Froehner et al., 1977a; Shorr et al., 1978) and to assay the binding of radioactive ligands to the receptor (Mittag et al., 1976; Hall and Reiness, 1977).

The property of the AChR to bind lectins has been used in this study to demonstrate the existence and separation of two distinct populations of α-BuTx binding sites in solubilized AChR extracted from denervated rat hindlimb muscle. The 41% maximal inhibition of toxin binding to receptors preincubated with soluble Con-A (Fig. 2) suggests the presence of two forms of toxin binding sites, each comprising approximately half of the total number of sites, one sensitive and the other not sensitive to Con-A inhibition of toxin binding. This finding is in agreement with that of Meunier et al. (1974), who demonstrated a partial inhibition by Con-A (60%) of α-neurotoxin binding to a highly purified receptor preparation extracted from the electric organ of Electrophorous electrius. They suggested that incomplete inhibition of toxin binding might be due to the heterogeneity of the carbohydrate moiety of the receptor. However, Almon and Appel (1976) reported that 0.1 μM Con-A completely inhibited α-BuTx binding to solubilized receptor extracted from denervated rat hindlimb muscle.

The blockade of toxin binding to AChR produced by Con-A is apparently irreversible. No reversal of inhibition is obtained after 24 hours incubation of Con-A treated receptors with α-BuTx at concentrations greater than 60 times its K_{Dapp} (Fig. 7). Thus, there appears to be no dissociation of Con-A from the receptor during this time and the blockade of toxin binding by Con-A is for practical purposes irreversible. Furthermore, as shown in Fig. 2, the inhibition of toxin binding produced by Con-A occurs only if the receptors have not been labelled with toxin. Such findings could result from either steric or allosteric inhibition of toxin binding by the interaction of Con-A with the AChR.

Steric hinderance directly at the toxin binding site or from a neighboring site (i.e., irreversible site-directed inhibition) is conceivable since, at pH 7.4, Con-A exists as a tetramer of 110,000 MW (Sumner et al., 1938). The binding of such a large molecule near the toxin binding site could easily deny toxin access to the receptor, a view also held by Almon and Appel (1976). An allosteric effect mediated by a conformational change induced by Con-A binding cannot be ruled out. Changes in the conformation of the receptor complex upon binding ligands have been reported for solubilized and membrane bound AChR extracted from Torpedo californica (Witzeman and Raftery, 1978). They suggested that conformational changes at the 40,000 dalton subunit (i.e., that subunit known to bind α-BuTx) upon cholinergic ligand interaction causes further intramolecular structural changes that involve subunits of higher molecular weight. These higher molecular weight subunits are therefore thought to belong to a supramolecular complex of interacting polypeptides asso-

ciated with the postsynaptic membrane. Similar results suggesting major conformational changes have been found in this study using AChR extracted from denervated rat muscle. Unlabelled AChR can readily be eluted from spheroidal hydroxylapatite columns with 150 mM Na/PO_4 buffer, as observed in the receptor preparations (see Methods), but toxin labelled receptors bind so tightly that they cannot be eluted with Na/PO_4 or $Na/K/PO_4$ buffer at concentrations up to 2 M (Mittag, Massa and Gross, unpublished). Toxin itself does not bind to HTP. The same effect has been noted for binding of AChR to Con-A-Sepharose and Lens culinaris-Agarose affinity columns. Unlabelled receptors can be partially eluted with mannose from Con-A and LCA columns, but toxin labelled AChR cannot (Mittag and Gross, unpublished). These differences in the interaction of AChR with lectins and HTP may result from conformational changes in the receptor induced by α-BuTx binding.

It is not known presently whether the binding of ligands (such as Con-A or antibodies) to the higher molecular weight subunits of the receptor affects cholinergic ligand binding at the 40,000 dalton subunit. Also, it is not known to which subunit(s) Con-A binds. It is possible that Con-A binding to the 40,000 dalton subunit and/or one (or more) of the higher molecular weight subunits induces a conformational change at the toxin binding site that results in blockade of toxin. An allosteric mechanism for the Con-A inhibition of toxin binding is not unreasonable, especially if one considers the AChR to be a regulatory protein controlling the flux of ions across the postsynaptic membrane. Allosteric inhibition of enzymes which catalyze the rate limiting step in a multi-step metabolic pathway is a well

known phenomenon (Piszkiewisz, 1977).

The present experiments do not allow a distinction to be made between these two possibilities, i.e., between steric or allosteric inhibition. At first glance, the results of the immunoprecipitation experiments using anti-Con-A appear to indicate that Con-A is capable of binding to labelled receptors in the same manner as unlabelled receptors (Fig. 2 and 3), suggesting that Con-A and toxin are not mutually exclusive and that the inhibition of toxin binding results from a Con-A induced allosteric alteration of the receptor. However, as will be discussed below, the receptor possesses several Con-A binding sites and it is therefore possible that one of these sites could correspond to the toxin binding site (or a closely neighboring site) for those receptors whose toxin binding capacity is affected by Con-A.

Comparison of the apparent equilibrium binding of toxin to AChR pre-treated with normal saline versus Con-A indicates not only a decrease in the total toxin binding capacity of the Con-A treated receptors (Fig. 6 and 7), but also an increase in the apparent K_D of those receptors still capable of binding toxin, i.e., Con-A-receptor complexes (Fig. 7). Since it appears that two populations of receptor are present based on differences in response to soluble Con-A, the question, arises whether the increase in apparent K_D of the Con-A-receptor complexes which are still able to bind toxin is due to the elimination of one class of receptors with a lower K_D from the toxin binding reaction. Alternatively a conformationally induced change may decrease the affinity of the Con-A-receptor complexes for toxin. This question cannot be resolved using equilibrium binding techniques

and will be temporarily deferred to introduce discussion of the kinetics of toxin binding to receptors pretreated with saline/or Con-A.

The fact that approximately half of the toxin binding sites could be inhibited by soluble Con-A and that the receptor is a glycoprotein suggested that the two forms of binding sites might be separable using lectin affinity column chromatography. Lens culinaris-Agrose (LCA) was chosen for this purpose in preference to Con-A-Sepharose. Although Con-A and Lens culinaris share the same carbohydrate specificity, i.e., α-D-mannose and α-D-glucose residues (Lis and Sharon, 1973; Hayman et al., 1973), Lens culinaris binds these residues in a more reversible manner (Kinzel et al., 1976). Comparison of the elution of receptors from LCA and Con-A-Sepharose affinity columns confirms this point (Fig. 22). The less tightly bound receptors are eluted from LCA within 12 hours with a .5 M α-methyl-mannoside solution, whereas elution from Con-A-Sepharose requires several days (Mittag and Gross, unpublished).

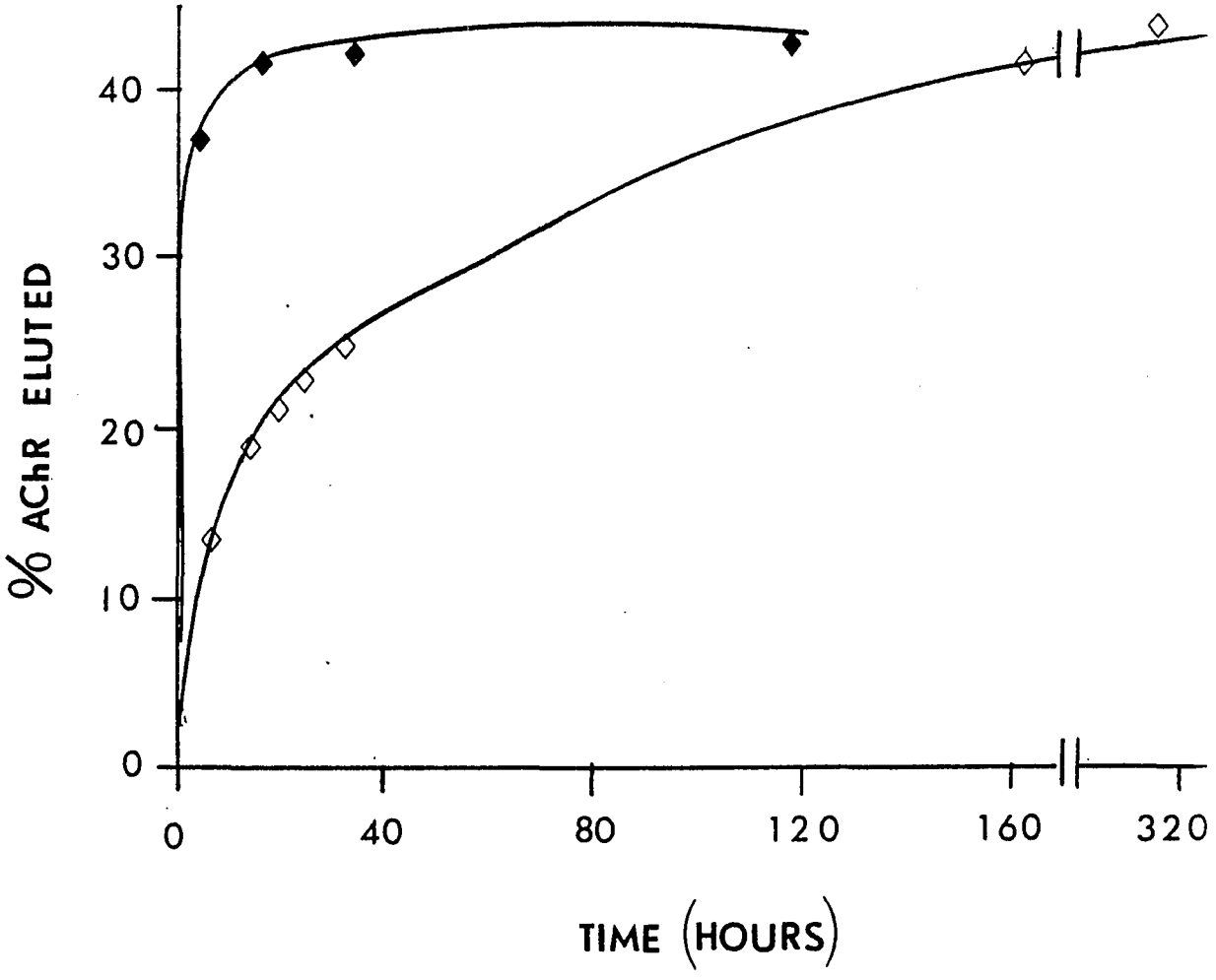
LCA affinity column chromatography of hydroxylapatite purified receptors results in the elution of 52% of the applied receptors; the remainder are tightly bound. The eluted receptors appear to be homogeneous, as all are susceptible to inhibition of toxin binding by soluble Con-A, as contrasted with only half of the hydroxylapatite prepared receptors (Fig. 8). Further evidence confirming the homogeniety of the LCA purified receptors was obtained by comparing the kinetics of toxin association and dissociation of LCA and hydroxylapatite AChR preparations.

The binding of toxin to HTP prepared AChR occurs by two kinetical-

Figure 22. HTP prepared AChR were recirculated through LCA or Con-A affinity columns for 24 hours at $4^{o}C$. Receptors bound to these columns were eluted with 0.5 M α-D-mannose and 1% Triton X-100 onto HTP columns and labelled with ^{125}I-α-BuTx. The maximum percent AChR eluted from the LCA column (closed diamonds) was 43%, while 40% was eluted from the Con-A affinity column (open diamonds). (Diagram courtesy of T.W. Mittag and S. Gross).

Figure 22

Purification of HTP Prepared AChR Using Con-A-Sepharose or Lens culinaris Agarose Affinity Chromatography



ly distinct processes. This can be demonstrated both by an evaluation of the kinetics of association (Fig. 4) as well as by an evaluation of the kinetics of toxin-receptor complex dissociation (Fig. 10). However, the LCA purified AChR have only one association rate constant (Fig. 9), and one dissociation rate constant (Fig. 11) for toxin. In both cases, the rate constants found for LCA purified AChR correspond to the fast associating and dissociating rate constants found for one of the two binding sites observed in the HTP preparations. Since LCA purified receptors are homogeneous with respect to toxin binding, it would appear that the fast associating and dissociating binding sites observed in the hydroxylapatite receptor kinetics experiments correspond to the same population of binding sites, i.e., these sites bind and dissociate toxin faster than the other population of binding sites. Brockes and Hall (1975a) reported similar findings. The K_D's for these two populations of binding sites found in the HTP receptors preparation as determined from the results of the kinetic experiments, are .303 nM and .669 nM (Table 13). The K_D of the LCA purified receptors is .221 nM.

These findings are in accord with those found by Brockes and Hall (1975a). They reported association rate constants of 3.1 x $10^5 M^{-1}s^{-1}$ and 1.2 x $10^5 M^{-1}s^{-1}$ at 37°C for solubilized receptors extracted from denervated rat diaphragm and that the two forms of binding sites were present in approximately equal amount. The corresponding dissociation rate constants are 6.3 x $10^{-5}sec^{-1}$ and 1.9 x $10^{-6}sec^{-1}$, respectively. The dissociation constants are, therefore, .2 nM and .02 nM, respectively. Although the data is not presented, Brockes and Hall (1975a) state that Scatchard analysis of equilibrium

Table 13

Estimates of Dissociation Constants (K_D) for the Toxin Binding Components found in HTP and LCA Prepared AChR

Constant	HTP AChR fast component	HTP AChR slow component	LCA AChR
$k_{-1}(sec^{-1})$	2.49×10^{-5}	2.34×10^{-6}	3.12×10^{-5}
$k_1(M^{-1}sec^{-1})$	8.23×10^{4}	0.35×10^{4}	1.18×10^{5}
calculated $K_D(nM) = \frac{k_{-1}}{k_1}$	.303	.669	.221
experimental $K_D(nM)$	.78	.78	-

Values for all association (Fig. 4 and 9) and dissociation (Fig. 10 and 11) rate constants were determined experimentaly, as was the "experimental" K_D for HTP prepared AChR (Fig. 6).

binding data resulted in two intersecting straight lines, also suggesting the presence of two classes of binding sites. Similar experiments conducted in the present study (Fig. 7) indicate only one apparently homogeneous population of toxin binding sites. Almon et al. (1974b) also observed only 1 population of binding sites (K_D = 1.3 nM) in a similar AChR preparation using solely equilibrium binding experiments.

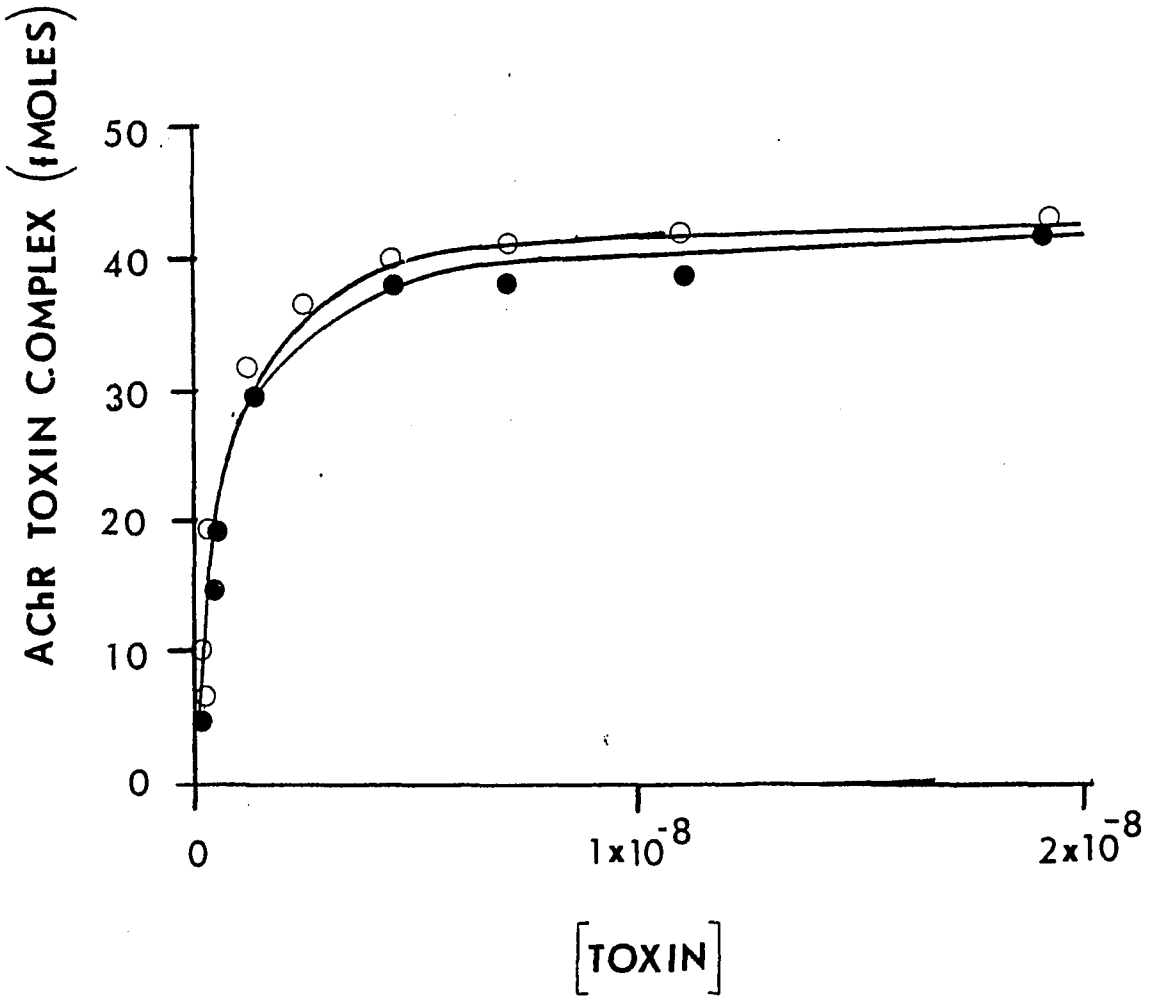
This apparent discrepancy between the results of the kinetic and equilibrium experiments is explained by the fact that under the conditions used in the equilibrium studies, and as verified by the kinetic studies, the two forms of toxin binding sites have apparent K_D's that are not sufficiently different to be clearly distinguished. Note that the K_D's reported by Brockes and Hall (1975a) differ by a factor of ten, but those found in the present study differ by a factor of only 2.2. Brockes and Hall conducted their experiments at 37°C, whereas the present observations were made at 4°C. The change in temperature appears to have affected one form of toxin binding sites more than the other and may explain why Brockes and Hall find two components in their equilibrium studies. This point is clarified in Fig. 23, which is a comparison of the experimental equilibrium binding curve and a calculated curve constructed using the K_D's for the two binding sites as determined by the kinetic data (Table 13). The contribution of both components at a given toxin concentration is summed and the resulting curve is analyzed in a manner similar to the experimental data. It is evident that computer analysis of the two component calculated model gives very similar results to the experimentally derived values (K_D calculated = .44 nM and K_D experi-

Figure 23. The K_{Dapp} obtained experimentally (open circles) for the equilibrium binding of α-BuTx to AChR (see Fig. 6) is 0.59 nM. The K_{Dapp} obtained using a two component model based on the results of the kinetics data (Table 13) is 0.44 nM (closed circles).

Figure 23

Apparent Equilibrium Binding of ^{125}I-α-BuTx to AChR

Comparison of Experimental and Calculated Binding Curves



mental = .59 nM). It would appear, therefore, that kinetic methods are more appropriate for studying the heterogeneity of the AChR. Furthermore, one must question the use of equilibrium studies to study the receptor when using very slowly reversible ligands such as α-BuTx. In such cases equilibrium studies are not practical since equilibrium is not necessarily reached until receptor saturation is achieved (Colquhoun and Rang, 1976; Brockes and Hall, 1975a; Raftery et al., 1976).

The kinetics of toxin binding to the remaining HTP prepared receptors which were not blocked by Con-A pretreatment indicates that the Con-A blocked receptors are those which have toxin sites with fast kinetics (Fig. 5), as the association rate constant of the remaining receptors corresponds to the slow binding sites of the control AChR. The possibility that Con-A blocks approximately half of both fast and slow toxin binding sites and converts the remaining fast sites to slow sites, while unlikely, cannot be ruled out based solely on this experiment. However, the 100% inhibition of toxin binding to LCA purified AChR, which corresponds to the population of AChR with fast kinetics of toxin binding, supports the suggestion that only the fast toxin binding sites in the HTP prepared AChR are blocked by Con-A.

Although these kinetics experiments allow discrimination of two distinct toxin binding sites, one of which is susceptible to inhibition by soluble Con-A, they do not aid in determining the nature of the Con-A induced inhibition of toxin binding mentioned earlier in the discussion of the equilibrium binding of toxin to Con-A pretreated AChR. The equilibrium experiments do not show the presence

of two kinetically distinct toxin binding sites because the apparent K_D's of these sites are too similar, and the data can be described by a single population of toxin binding sites equally well. Thus, it is conceivable that inhibiton of one of the two kinetically distinct toxin binding sites by soluble Con-A could result in an altered apparent K_D in the equilibrium binding studies, while still maintaining the appearance of a homogeneous receptor population. Such a change could occur without any effects on the receptors still capable of binding toxin. However, the kinetics studies indicate that the toxin binding sites with fast rates of association and dissociation (lower affinity) are blocked by Con-A. One would therefore expect the apparent K_D to decrease, not increase, as was observed in the equilibrium binding of toxin to Con-A pretreated AChR. If Con-A does indeed inhibit only those toxin binding sites with fast kinetics of toxin binding without altering the kinetics of association to the slow kinetic binding sites, an increase in apparent K_D could only result from alteration (increase) of the rate of dissociation of Con-A-receptor-toxin complexes. This implies that in addition to inhibiting toxin binding to one class of toxin binding sites, Con-A also induces conformational changes in the other class of toxin binding sites.

An interesting aspect of the interaction between Con-A and the AChR is the magnitude of the Hill coefficients for the inhibiton of toxin binding (1.9 ± 0.3), and complex formation of labelled (5.5 ± 0.7) and unlabelled (4.3 ± 0.7) Con-A treated AChR with anti-Con-A. Hill coefficients of this size indicate positive cooperactivity in the binding of multiple molecules to the receptors or cross-linking.

Con-A also stimulates membrane bound magnesium-dependent ATPase of mouse neuroblastoma cells (Traficante et al., 1978) and liver plasma membrane (Riordan et al., 1977) through positive cooperative processes and has also been reported to exhibit positive cooperativity in binding of Con-A neuroblastoma cells (Rosenberg and Charalampous, 1977), rat thymocytes (Karsenti et al., 1977), and Chinese hamster ovary cells (Ceri and Wright, 1977). For the interaction of lectins with cell surfaces, positive cooperativity has been explained either by an increase in the affinity of receptors for the lectin (Ceri and Wright, 1977; Rosenberg and Charalampous, 1977; Nicholson, 1976; Sonenberg, 1971) or by an increase in the number of available binding sites caused by the unmasking of "cryptic" receptors (Karsenti et al., 1977; Bornens et al., 1976). These changes may be the result of either conformational changes in membrane components or of their redistribution in the membrane facilitated by the fluid character of the membrane (Singer and Nicolson, 1972). Schechter et al. (1976) and Prujansky et al. (1978) demonstrated the necessity of lectin multivalency for stimulation of mouse lymphocytes, suggesting that the triggering signal for lymphocyte stimulation requires cross-linking and/or clustering of lectin-receptor complexes. Cross-linking of lectin receptors on different cells is also evident in the agglutination of erythrocytes (Sumner et al., 1936a).

The positive cooperativity exhibited by Con-A in the present study could result from cross-linking of carbohydrate moieties on different receptors by Con-A. This would not be surprising since at pH 7.4 Con-A is a tetramer, with each subunit capable of binding carbohydrates (Becker et al., 1971). Furthermore, Mattson and

Heilbronn (1975) have demonstrated that carbohydrates contribute 10% of the molecular weight of the AChR 80% of which are α-D-mannoside residues. Cross-linking of Con-A receptor sites on the same AChR and between two or more AChR's is, therefore, possible.

However, it seems unlikely that cross-linking plays a role in the Con-A inhibition of toxin binding or in the complexing of Con-A to toxin labelled receptors. As shown in the experiments involving Con-A and MG-I binding to toxin labelled receptors (Table 11), Con-A is more likely to cross-link AChR with an IgG molecule rather than another receptor, despite the fact that the affinity of IgG for Con-A is lower than the affinity for the receptor for Con-A. (The Con-A-IgG interaction is inhibited with 10 mM α-methyl-D-mannoside, whereas 50 mM α-methyl-D-mannoside is required to inhibit the AChR-Con-A interaction, as noted by Brockes and Hall, 1975b.) Con-A inhibits toxin binding to HTP prepared AChR with a Hill coefficient of 1.9 ± 0.3 and apparent K_D (based on the ED_{50}) of 1 μM. The apparent K_D for the binding of Con-A to toxin labelled receptors is also 1 μM but the Hill coefficient is 5.5 ± 0.7. Since the apparent affinity of the receptor for Con-A does not change upon binding toxin, we must assume, in the absence of cross-linking, that toxin binding causes a conformational change in the AChR and reveals cryptic Con-A receptors, as indicated by the change in the Hill coefficients. A similar change in the Hill coefficients was observed when these experiments were repeated using LCA purified AChR.

To summarize, the interaction of hydroxylapatite prepared receptors with Con-A clearly shows the existence of two distinct populations of toxin binding sites. Do these two populations of binding

sites represent subunits of the same molecule or two separate molecular entities? This question is answered by the results of experiments on the interaction of soluble Con-A with a fraction of the receptor further purified on Lens culinaris-Agrose (LCA). LCA affinity chromatography of hydroxylapatite prepared AChR results in the elution of 50% of the applied receptors; the remainder are tightly bound. The eluted receptors appear to be homogeneous, as they have only one association and dissociation rate constant for toxin binding (which are similar to the fast associating and dissociating component observed in hydroxylapatite prepared AChR) and all are susceptible to inhibition of toxin binding by soluble Con-A, as contrasted with only half of the hydroxylapatite purified receptors. It is clear that LCA affinity chromatography with .5 M α-methyl-mannoside resulted in the physical separation of two molecular species of toxin binding sites.

The simplest model that accounts for these results is two, separate, independent molecular species of receptor, characterized by differences in kinetics of toxin binding and different glycoprotein moieties. One species has a fast associating and dissociating toxin binding sites(s) that is susceptible to block by soluble Con-A, if it preceeds toxin labelling, and its lectin binding is weaker compared to the other species. The other class of molecules has a slow associating and dissociating toxin binding site(s) that is unaffected by Con-A binding. The only apparent inconsistency in this model is that unlabelled receptors bound to Con-A or LCA affinity columns are all capable of binding toxin and can be fully labelled on the gels (Table 2). One would expect only half of these receptors

to bind toxin when bound to these lectins, based on the inhibition findings with soluble lectin pretreatment. It is possible that when soluble Con-A (or LCA) binds to the receptor, the receptor undergoes a conformational change that results in the observed inhibition of toxin binding to one species of the receptor, as discussed earlier. This conformational change might not be possible when the receptors are bound to immobilized lectin. Alternatively, the Con-A binding sites involved in binding to the affinity columns may be different from those causing inhibitions of toxin binding which are inaccessible to Con-A molecules bound to a matrix but not the soluble lectin. The possibility of multiple Con-A binding sites on the receptor was discussed earlier.

Reports of receptor purification using LCA, Con-A and/or cobra toxin affinity chromatography support this model. Almon and Appel (1976) reported 40% to 60% elution of the receptors applied to a Con-A-Sepharose affinity column. Froehner et al. (1977a) eluted 33% of the receptors applied to a Con-A-Sepharose affinity column with 1.8 column volumes of a .4 M α-methyl-mannoside solution. Better recovery might have been achieved with more extensive washing or recirculation of the mannoside solution. The results of their studies thus apply to a subpopulation of AChR. These workers did not check for receptors remaining bound to the Con-A affinity column.

Patrick et al. (1973) have suggested that α-cobra toxin affinity chromatography of AChR may result in separation of two species of receptor since the yield from such precedures is usually 40% to 60% for receptors extracted from Torpedo (Schmidt and Raftery, 1972), rat muscle (Lindstrom and Patrick, 1974), and Electrophorous (Karlsson

et al., 1972). Toxin affinity columns might be expected to select a subpopulation of AChR if the toxin binding kinetics differed between the two (i.e., differences in association and dissociation rates), as seems to be the case according to the kinetic studies presented earlier. Shorr et al. (1978) also obtained approximately 50% yield of AChR using α-neurotoxin affinity chromatography of solubilized denervated cat hindlimb muscles. Further purification of these receptors using LCA affinity chromatography gave a 90% yield, suggesting that α-neurotoxin selected for a homogeneous population of toxin binding sites.

The presence of anti-receptor antibodies in the sera of patients with myasthenia gravis (M.G.) has been demonstrated by numerous workers (Bender et al., 1975; Almon and Appel, 1974a; Lindstrom et al., 1976a; Mittag et al., 1976; Fambrough et al., 1973). Antibodies capable of complexing with α-BuTx labelled receptors have been demonstrated directly, by immunoprecipitation, in the sera of more than 90% of such patients (Lindstrom et al., 1976a; Appel et al., 1975; Mittag et al., 1976). Antibodies have also been detected by a variety of other methods, such as inhibition of α-BuTx binding to solubilized receptors (Fambrough et al., 1973; Almon et al., 1974a; Mittag et al., 1976) and intact muscles (Bender et al., 1975, 1976a, 1976b), interference with receptor binding to Con-A-Sepharose (Mittag et al., 1976), and acceleration of the rate of toxin labelled receptor degradation *in vitro* (Appel et al., 1977; Heinemann et al., 1977, 1978; Kao and Drachman, 1977a) and *in vivo* (Stanley and Drachman, 1978). The increased rate of receptor degradation was noted for both innervated and denervated rat receptors treated with myasthenic

sera (Heinemann et al., 1978, and Reiness et al., 1978). The role of the antibodies in the pathogenesis of myasthenia gravis is uncertain at present and it is not known what event(s) initiates their production.

Although these differing reactivities with AChR have been ascribed to myasthenic antibodies, heterogeniety of these antibodies was not considered until suggested by Mittag et al. (1976). These authors found that 10 of 15 myasthenic sera inhibited the binding of solubilized AChR extracted from denervated rat hindlimb muscle to Con-A-Sepharose. In addition, 13 of 15 sera were positive for anti-receptor antibodies (as determined by immunoprecipitaiton of toxin-labelled receptor complexes) and only 1 of 15 sera caused inhibition of toxin binding to membrane bound receptors. Some sera with high titers of anti-receptor antibody did not appreciably block receptor binding to Con-A-Sepharose, while others, with low anti-receptor titers, exhibited extensive blocking of receptor binding to Con-A. These results suggested the presence of as many as three antibody types in the sera of myasthenic patients. The titer of these antibodies varies independently from one patient to the next, and it is therefore possible for a patient to have one or all three antibodies in varying titers.

The results screens carried out on the sera and IgG in the present study agree very well with those of Mittag et al. (1976). Ninety-one of 187 (49%) myasthenic sera tested were positive for the antibody inhibiting receptor binding to Con-A-Sepharose (MG-S), 19 (10%) for toxin blocking antibody (MG-B) and 11 (6%) had both MG-S and MG-B, as determined by the Con-A/HTP cascade assay method. The

presence of immunoprecipitating antibodies was not determined for this group of myasthenic sera. However, as seen in the evaluation of myasthenic IgG prepared from these sera, the titers of MG-S, MG-B and MG-I appear to vary independently of one another from patient to patient. This confirms the presence of three separate antibody types in myasthenic sera directed against the nicotinic receptor.

Interaction of myasthenic antibodies with AChR strongly suggests the presence of multiple forms of α-BuTx binding sites. Factor I (MG-I) forms complexes with labelled as well as unlabelled AChR which immunoprecipitate when the MG-I-AChR-toxin complex is exposed to anti-human IgG (Fig. 12). Complexing of this antibody to receptors has no effects on the binding of toxin to AChR. It alters neither the total binding capacity of the receptor (Fig. 12) nor the kinetics of toxin binding to receptors (Fig. 13). This is the class of antibody most common among myasthenic patients and likely the antibody measured when determining the titer of myasthenic anti-receptor antibodies in diagnostic assays (Lindstrom et al., 1976a, 1978). The MG-I sites on the receptor are distinguished from the Con-A binding sites; namely MG-I neither affects Con-A-Sepharose binding of labelled AChR nor does it interfere with soluble Con-A binding to labelled AChR (Table 11). Although the results of studies on the interaction of this antibody with AChR do not demonstrate the heterogeneity of the toxin binding sites, they do establish that separate sites exist on the AChR for toxin, Con-A and MG-I, which is the most common type of antibody found in myasthenic IgG preparations.

The interaction of AChR and MG-B, another antibody present in myasthenic sera, does however support the concept of receptor hetero-

geneity. Incubation of toxin labelled hydroxylapatite prepared receptors with MG-B does not displace toxin bound to the receptor. However, incubation of unlabelled AChR with MG-B results in a dose-dependent inhibition of toxin binding (Fig. 17). The maximum inhibition of toxin binding is 30 ± 1.9% and the Hill coefficient for the inhibition is 1.95. As previously discussed, a Hill coeffiecient of this magnitude indicates positive cooperativity in the binding of MG-B to those receptors affected by MG-B. MG-B, like Con-A, produces an insurmountable inhibition of toxin binding which is apparently irreversible. As previously noted, the inhibition occurs only if the receptors are treated with MG-B prior to labelling with α-BuTx. Furthermore, as observed in the apparent equilibrium binding of toxin to MG-B treated AChR (Fig. 18), the inhibition is not overcome by labelling for 24 hours at toxin concentrations as high as 60 nM (greater than 60 x K_{Dapp}). This indicates that toxin cannot displace MG-B from the AChR. Thus, the inhibition produced by MG-B resembles that induced by soluble Con-A, and may occur by steric or allosteric mechanisms. However, using HTP prepared receptors, the maximum inhibition of toxin binding by Con-A was 41% as contrasted to 30% by MG-B. MG-B also differs from Con-A in that prior labelling of receptors with toxin precludes MG-B binding as evidenced by the lack of immunoprecipitation by anti-human IgG of labelled receptor that had been incubated with MG-B isolated from patient M174 using Protein-A-Sepharose affinity chromatography.

In order to determine whether or not Con-A and MG-B inhibited toxin binding to the same set of toxin binding sites, hydroxylapatite prepared AChR were pretreated with soluble Con-A followed by MG-B,

and vice-versa, and then subsequently incubated with ^{125}I-α-BuTx. In each case, toxin binding was reduced to 30% of control. These results show that the effects of Con-A and MG-B are additive and suggest binding to mutually exclusive sites. The results of other experiments established that Con-A and MG-B binding sites correspond to different toxin binding sites.

As shown previously, Con-A blocks 40% of the toxin binding sites present in HTP prepared AChR (Fig. 2) and 100% of the sites in LCA purified AChR (Fig. 8). However, MG-B does not affect toxin binding to LCA purified AChR. Since it has been established that LCA purified AChR contain only one set of toxin binding sites which are characterized by faster association (Fig. 9) and dissociation (Fig. 11) rate constants, it can be inferred that MG-B reacts only with the toxin binding sites characterized by the slower rate constants. Further, since these "slow" toxin binding sites constitute approximately 60% of the total binding sites, a maximal inhibition of 30% by all but one MG-B IgG suggests that there are at least two subpopulations of slow α-BuTx binding sites. Thus, the heterogeneity of toxin binding sites in HTP purified AChR appears to be more complex than initially suggested by the results of the lectin (Con-A) binding studies. However, until it is possible to physically isolate a receptor preparation containing only slow toxin binding sites and study directly their interactions with MG-B, these conclusions must be held as tentative.

MG-I, MG-B and MG-S found in myasthenic sera are assumed to be antibodies in the immunoglobulin-G class. Namely, these factors are immunoprecipitated using anti-human IgG (Lindstrom, 1976;

Almon, and Appel, 1974a; Mittag et al., 1976, 1978). In addition the factors are associated with the IgG fraction of myasthenic sera prepared using several different techniques (Bender et al., 1976a; Mittag et al., 1976, 1978). Purification of these factors using Protein-A-Sepharose affinity chromatography provides the strongest evidence that these factors are indeed antibodies of the IgG class. The interactions of Protein-A-Sepharose purified IgG fractions with AChR paralleled those found using DEAE-Sephadex purified IgG fractions (Table 9) for five of six sera examined. This finding indicates that the myasthenic factors are of the immunoglobulin class G, in particular, IgG subclass 1 and 2. Protein-A-Sepharose specifically binds the Fc region of IgG subclasses 1, 2 and 4 (Kronvall and Williams, 1969). In addition, little, if any IgG_4 is found in IgG fractions prepared using DEAE-Sephadex chromatography (Skvaril et al., 1970). Since the DEAE preparations give essentially the same results as the Protein-A preparations, the myasthenic anti-receptor antibodies would appear to be in subclass 1 and/or 2. This is further supported by the finding that a Protein-A purified IgG fraction of MG-B prepared from a DEAE purified IgG fraction of MG-B interacts with AChR in a manner identical to the DEAE prepared fraction (Table 9 - M250).

There was one significant exception to the generalization that DEAE and Protein-A preparations produce the same result. IgG prepared using Protein-A chromatography from the serum of patient M174 resulted in an MG-B preparation capable of inhibiting 100% of the toxin binding to HTP prepared receptors, which contain both fast and slow toxin binding sites (Fig. 20). No reversal of toxin binding was

observed when receptors were prelabelled prior to incubation with MG-B-174. No immunoprecipitation of toxin-labelled receptors with anti-human IgG was observed for receptors labelled prior to or after incubation with MG-B-174. These results indicate that MG-B inhibits toxin binding by interacting with AChR directly at, or near, the toxin binding sites. Complete inhibiton of toxin binding was not observed in any of the other MG-B preparations whether prepared using DEAE or Protein-A chromatography. The very limited amount of this patient's serum precluded preparation of a DEAE purified IgG fraction for comparision; nor has it been possible to obtain other samples of sera from this patient.

It is difficult to explain fully the basis of this unusual behavior. However, one can postulate that this patient, unlike others, possessed a form of MG-B which was directed against all toxin binding sites, rather than a specific subpopulation of sites. Or alternatively, this patient had multiple forms of MG-B, each directed against a single population of toxin binding sites. Additionally, the Fc region of this MG-B may have had a higher affinity for Protein-A than other IgG's. The purification process would have selected for this particular subpopulation of IgG, resulting in a highly concentrated, potent MG-B preparation.

Toxin blocking antibody has been reported by several groups. Bender and his colleagues (Ringel et al., 1975; Bender et al., 1976) demonstrated that normal human muscle specimens could not bind any α-BuTx when incubated with either myasthenic serum or IgG. Incubation with normal serum or IgG had no effect on toxin binding. Zurn and Fulpius (1976) supported these findings by showing that the

postjunctional membrane of intact mouse neuromuscular junctions were readily accessible in vivo to circulating molecules the size of IgG.

Using Protein-A affinity chromatography, Lefvert and Bergstrom (1977) have isolated an IgG_3 from myasthenic lymph capable of blocking 60% of the toxin binding to membrane bound AChR extracted from the electric organ of Torpedo marmorata. Although this would appear to disagree with the present finding that MG-B appears to be an IgG_1 or IgG_2, it should be recalled that Torpedo AChR are immunologically different from denervated rat receptors with respect to their reactivity with human myasthenic antibodies (Lindstrom et al., 1978; Lennon, 1976). It is significant that roughly half of the Torpedo receptors are susceptible to toxin block, indicating the presence of multiple forms of AChR.

The presence of a toxin-blocking antibody in the serum of at least five and possibly eleven out of fifteen patients with myasthenia gravis has been demonstrated using solubilized denervated rat muscle AChR (Appel et al., 1975; Almon et al., 1974a). Fifty percent maximum inhibition of toxin binding was observed and assumed to be allosteric in nature, i.e., that the blocking antibody binds at a site other than the toxin binding site. This was based on the ability of anti-human IgG to immunoprecipitate labelled AChR when receptors were either labelled or unlabelled prior to treatment with IgG (Almon and Appel, 1975). The implication was that blocking antibody could bind to both labelled and unlabelled AChR, but inhibition resulted only in the unlabelled case. The observations reported by Almon and Appel (1975) would appear to be in conflict with the results obtained in the present study. However, it should be noted

that these investigators only employed immunoprecipitation to measure antibody-receptor interactions and used a relatively small patient population. In the present study, a larger patient population was used and the interactions of anti-receptor antibodies with AChR was studied using several different techniques. Thus, it has been possible to clearly show that there are at least three forms of IgG present in the sera of myasthenic patients that are directed toward the nicotinic AChR. Of these, MG-B and MG-I almost always occur together, but their titers are not co-variant. Herein, MG-B is readily distinguished from MG-I by the use of the HTP assay in conjunction with immunoprecipitation. Thus the observations of Almon and Appel (1975), while correct, must be reinterpreted based on the observations made in this study. Using only immunoprecipitation, they could not distinguish between MG-B and MG-I when present together. In the present study, we were very fortunate to find one patient, M174, who possessed only MG-B type antibodies. Using the Protein-A purified IgG fraction from this serum, it was unequivocally shown that MG-B and toxin compete for the same site, namely the binding of one of these essentially irreversible ligands precludes the binding of the other.

The third antibody examined, MG-S, interferes with the binding of labelled receptors to Con-A-Sepharose (Fig. 15). The receptors which are prevented from binding to Con-A-Sepahrose are recovered on HTP columns when the cascade assay method is used. The total binding capacity of the receptor is unaffected by MG-S (Table 8). The presence of MG-S is indicated by a decrease in the amount of labelled receptor bound to Con-A and a corresponding increase in that

bound by HTP. Thus, the Con-A/HTP ratio is seen to decrease from a control value of 7 to a minimum value of approximately 1. The maximum inhibition of receptor binding to Con-A was approximately 50%. This suggests there are two populations of receptors differing in their interaction with Con-A-Sepharose when complexed with MG-S. Similar results have been reported using whole serum of myasthenic patients (Mittag et al., 1976).

It seemed reasonable to question whether the 50% of receptors unable to bind to Con-A-Sepharose following treatment with saturating amounts of MG-S corresponded to one or another of the kinetically distinct toxin binding sites present in the AChR population. Kinetics of toxin binding to AChR preincubated with excess MG-S reveal that although MG-S does not alter the toxin binding capacity of the receptors, it does alter the kinetics of both fast and slow toxin binding sites (Fig. 16 and Table 3). The original kinetic experiments indicated that the fast toxin binding site corresponded to the 50% of the MG-S treated receptors remaining on Con-A and those receptors which were displaced were assumed to have slow toxin binding kinetics, although this was not measured directly (Mittag et al., 1978). However, a more careful analysis of the toxin binding kinetics of the displaced AChR shows that the binding sites remaining on Con-A as well as those displaced onto HTP, consist of a slow and a fast toxin binding component (Fig. 16). Taken alone, this could best be explained by two classes of receptor, each having fast and slow binding sites and differing carboyhydrate moieties. MG-S is able to discriminate them on the basis of their carbohydrate groups. At first approximation, such a simple model does not agree with the

results of experiments on the interaction of soluble Con-A and/or MG-B with hydroxylapatite and LCA purified AChR. These results showed two separable classes of AChR discriminated by their kinetics of toxin binding (fast and slow). However, a much more complex model is required to explain these results in conjunction with the MG-S experiments. The inhibition of toxin binding to LCA and HTP purified receptors by soluble Con-A showed that the fast and slow toxin binding sites were on separate species of the receptor. The MG-S experiments indicate that both the fast and slow toxin binding forms of AChR can be further subdivided by MG-S on the basis of inhibition of binding to Con-A-Sepharose. The Con-A binding sites affected by MG-S appear different from those which prevent toxin binding when complexed to soluble Con-A. The simplest explanation that accounts for all these findings is a model with four separate, independent receptor molecules characterized according to kinetics of toxin binding and differing Con-A binding sites. Approximately half of the molecules have fast kinetics of toxin binding, while the other 50% are slow. Soluble Con-A inhibits toxin binding to the fast toxin binding sites. Each half of the receptor is further divided into equal subgroups (each subgroup, therefore, represents roughly 25% of the total AChR) with a carbohydrate moiety which either binds MG-S thereby prevents the binding of the receptor to Con-A-Sepharose, or does not bind MG-S and therefore shows no displacement from Con-A-Sepharose in its presence.

Such a model is supported by the following experiments. Purification of AChR using LCA affinity chromatography results in a homogeneous receptor preparation having only fast toxin binding sites

which can be blocked by Con-A. Treatment of LCA purified receptors with MG-S prior to toxin labelling results in 50% displacement of toxin-receptor antibody complexes from their binding to Con-A-Sepharose (Table 10), indicating that the receptors with fast toxin binding sites can be separated into two subpopulations by MG-S. Thus, the behavior of LCA purified AChR is similar to hydroxylapatite purified AChR when treated with MG-S. Since MG-S produces a 50% inhibition of toxin-receptor complex binding to Con-A-Sepharose for LCA (fast toxin binding sites only) as well as HTP prepared receptors (fast and slow toxin binding sites) the slow sites also have two components which can be discriminated by MG-S. The subdivision of slow sites is further supported by the fact that MG-B apparently recognizes only 50% of the slow toxin binding sites. In addition to blocking half of the receptor binding to Con-A-Sepharose, the kinetics of toxin binding are significantly slowed by MG-S (Fig. 16) although the maximum toxin binding capacity is unaltered.

When hydroxylapatite purified AChR are labelled prior to treatment with a saturating concentration of MG-S, only 25% block of receptor binding to Con-A-Sepharose is observed (Fig. 15 and Table 10) as contrasted to 50% block when the order of addition is reversed, i.e., with MG-S prior to α-BuTx. In each case, the receptors blocked from the Con-A-Sepharose column are completely recovered on the HTP column of the cascade. This suggests that toxin binding somehow protects one of the two classes of molecules that would otherwise be displaced from Con-A-Sepharose (i.e., after exposure of unlabelled receptors to MG-S). Such protection probably occurs through a conformational change induced when the receptor binds toxin. Since the re-

ceptors are prelabelled with toxin, it is not possible to characterize the receptors displaced from Con-A-Sepharose in this case by their kinetics of toxin binding. When this experiment was repeated using LCA purified (fast toxin binding only) AChR, there was no difference in the displacement from Con-A-Sepharose between receptors labelled before or after treatment with MG-S. Fifty percent inhibition of receptor binding to Con-A-Sepharose occurs in both cases (Table 10). Thus, the 25% of the HTP prepared receptors containing (fast and slow toxin binding populations) protected by prelabelling with toxin from the displacement effect of MG-S represent half of the population with slow kinetics of toxin binding.

The proposed model of different forms of extrajunctional nicotinic receptors is more complex than those currently used to describe these receptors. Only one molecular species has been demonstrated by physical separation methods such as ultracentrifugation (Chiu et al., 1973), gel filtration (Brockes and Hall, 1975b) or isoelectric focusing (Brockes and Hall, 1975b). Since it is thought that one receptor molecule binds two to four toxin molecules (Karlin, 1977) and heterogeneity of toxin binding sites on the same molecule is not unreasonable, the kinetics data could fit into a one species model. Based on the separation and interaction of receptors with lectins, however, the two species model seems more reasonable, and a four entity model is required to explain the interaction of HTP prepared AChR with myasthenic IgG. Although physical separation techniques have yet to show gross differences in the AChR complex, the results of the present study show that major differences exist in antibody and lectin binding sites.

As noted by Raftery et al. (1976), the origin of receptor and the biological role of multiple receptor forms is unknown at present. Such differences are not due to differences between innervated and denervated receptors. Although innervated receptors persist in denervated muscle (Frank et al., 1976), they contribute only 5% to 10% of the total receptor content in the solubilized receptor muscle extracts (Almon et al., 1974; Berg et al., 1972; Miledi and Potter, 1971; Fambrough, 1970, Alberquerque and Thesleff, 1968). Differences in receptors found in membranes of fast and slow muscles could be responsible for these findings, as the preparations used in this study are mixed muscle preparations consisting of fast, slow and mixed muscle types. Almon et al. (1974b) have found very little difference in the K_D of fast versus slow muscles using equilibrium binding of α-BuTx. However, as discussed earlier, the ability to detect different forms of AChR by this method is doubtful, as discussed earlier. A detailed investigation of fast and slow muscles, studying the interactions of their receptors with Con-A and myasthenic antibodies, will be necessary to determine whether or not differences in these receptors are the source of heterogeneity in mixed muscle preparations.

Alternatively, these differences might result from minor alterations in the conformation of the receptor supramolecular complex. This proposal would not preclude differences between fast and slow muscle fiber types. It is well known that receptors extracted from electric fish (Karlin, 1977) as well as muscle (Froehner et al., 1977a, 1977b; Shorr et al., 1978) are composed of several subunits of differing molecular weights. To date, the only subunit ascribed any specific activity is the 40,000 dalton subunit, which has been

found to bind α-BuTx. All of the subunits found in receptors extracted from denervated rat muscle have been found to be antigenic when injected into rabbits, implying that all stimulate antibody production and bind antibody. However, the native receptor is capable of inducing greater antibody production than any of the subunits (Lindstrom et al., 1978b). It has not been determined which subunit binds Con-A. Furthermore, it has recently been demonstrated that the binding of cholinergic ligands to the 40,000 dalton subunit induces conformational changes in the higher molecular weight subunits in receptor extracted from Torpedo californica (Witzeman and Raftery, 1978).

Since these subunits were obtained from native receptors, it has been assumed that all receptors have the same subunit composition and that the receptors are homogeneous in the conformation these subunits assume in the supramolecular complex (Karlin, 1977). The molecular weight of the solubilized AChR complex is reported to be between 250,000 and 400,000 daltons (Rang, 1975). Since the present studies indicate very subtle differences in the four species of receptor, perhaps the observed heterogeneity results from receptors which have differing subunit compositions. The 40,000 dalton subunit would be common to all forms of the receptor as this is the only subunit known to bind toxin (Shorr et al., 1978; Karlin, 1977). The remainder of the complex could be composed of varying combinations of the remaining subunits. Such an arrangement would allow for receptors with approximately the same molecular weight, overall charge and possibly general conformation of the complex and would result in an inability to distinguish multiple forms of the

receptor using conventional separation techniques. Only very highly specific ligand-receptor interactions, such as those with Con-A and myasthenic antibodies, would be able to detect these subtle differences. It is noteworthy that immunological techniques are currently being used to study the thyrotropin (Smith, 1977) and insulin receptors (Flier et al., 1977). Perhaps even more interesting is that the antibodies used to study these receptors are obtained from the sera of patients with the autoimmune disorders Graves' disease and insulin resistant diabetes, respectively. The key to the pathogenesis and etiology of such disorders may lie in the immunological investigation of the receptors affected by these autoantibodies.

The next logical question is whether or not receptor homogeneity exists in preparations extracted from innervated muscles. The very low receptors yield from such preparations makes such studies difficult and, as a result, very little has been reported on innervated receptors. It is known that receptors extracted from innervated rat hindlimb muscle do not cross-react very well with myasthenic IgG (Almon and Appel, 1976b; Lindstrom, 1976; Lindstrom et al., 1978a). Presumably the antibody examined in these studies is MG-I and the present studies show that MG-I does not discriminate any of the four forms of receptor found in denervated receptor preparations. Whether or not MG-S and MG-B affect innervated receptors remains to be seen.

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