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Immunological studies on the structure and function of the nicotinic acetylcholine receptor in mammalian muscle

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IMMUNOLOGICAL STUDIES ON THE STRUCTURE AND FUNCTION OF THE NICOTINIC ACETYLCHOLINE RECEPTOR IN MAMMALIAN MUSCLE

by

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DISSERTATION

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TO MY PARENTS,

FOR THEIR LOVE, SUPPORT AND UNDERSTANDING.

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Immunological Studies on the Structure and Function of the Nicotinic Acetylcholine Receptor in Mammalian Muscle

Yong Gu

Summary

1. The specificity of the antibodies in the serum of a patient with myasthenia gravis for the α-bungarotoxin binding sites of the acetylcholine receptor (AChR) was examined using AChRs in the C2 mouse muscle cell line as a model. The antibodies were shown to be specific for one of the two toxin-binding sites that each receptor possesses, demonstrating that the two sites are immunologically distinct.

2. The effect of the antibodies in this myasthenic serum on the functional response of the receptor to cholinergic agonists was also examined using carbamylcholine-induced ^{22}Na uptake into C2 myotubes as a measure of the receptor function. The antibodies blocked the functional response of the receptor to carbamylcholine in a concentration-dependent manner. The relation between the inhibition of toxin binding and the inhibition of ^{22}Na uptake suggests that blocking one of the two binding sites by the antibodies completely impairs receptor function.

3. Antibodies specific for the γ, δ, and ε subunit, respectively, of mammalian muscle AChRs were developed using subunit-specific synthetic peptides as antigens.

4. Immunoprecipitation of solubilized AChRs with these antibodies demonstrated that anti-γ antibodies recognize AChRs of embryonic and denervated adult rat muscle but not those of normal adult muscle; anti-ε antibodies recognize AChRs from normal adult endplates, but are only weakly active against AChRs of denervated adult muscle and not active at all against embryonic AChRs; anti-δ antibodies are similarly active against AChRs of all sources.

5. Immunocytochemical staining with these antibodies demonstrated that AChRs at developing rat endplates switch from anti-γ immunoreactive to anti-ε immunoreactive during the first and second postnatal weeks; that endplates in denervated adult rat muscle retain their anti-ε immunoreactivity, but also become immunoreactive to anti-γ antibodies.

6. The junctional and extrajunctional regions of denervated adult muscles each contain both anti-γ and anti-ε immunoreactive AChRs. The ratio of the two types of AChRs in each region is about the same.

8. Using these antibodies and monoclonal antibodies for other subunits as probes, I have identified four (α, β, γ, and δ) subunits of mammalian muscle AChRs on immunoblots. The α and β subunits

contain only high-mannose N-linked oligosaccharides that are sensitive to endoglycosidase H, whereas the γ and δ subunits contain both high-mannose and complex types of sugars.

9. When AChRs from embryonic, neonatal, normal and denervated adult muscles were compared on immunoblots, the α, β, and δ subunits were identical in all four receptor preparations, with or without endoglycosidase digestion. The γ subunit was detected only in AChRs of embryonic, neonatal and denervated muscles, where their properties are identical. No band corresponding to the γ subunit was detected in AChRs of adult rat muscle.

10. I conclude from the experiments with subunit-specific antibodies that the spatial and temporal distribution of the γ- and ε-AChRs in developing and in denervated muscles corresponds to the distribution of AChRs with slow and fast channels, respectively, and that the developmental changes in the channel properties of the receptor arise from a change in the subunit composition of the receptor, in which the γ is replaced by ε.

Chapter 1

Molecular Structure and Developmental Changes of the Nicotinic Acetylcholine Receptor

An Introduction

The nicotinic acetylcholine receptor at the mammalian neuromuscular junction and at related synapses in the electric organs of *Torpedo* and *Electrophorus* is one of the most extensively studied neurotransmitter receptor proteins. Its functional as well as its biochemical and biophysical properties have been well characterized (McCarthy et al., 1986; Popot and Changeux, 1984; Stroud, 1983; Conti-Tronconi and Raftery, 1982, Karlin, 1980). In both mammalian and *Xenopus* muscles, the acetylcholine receptor at the neuromuscular junction undergoes several changes in its functional properties during development (Salpeter and Loring, 1985; Salpeter, 1987; Schuetze and Role, 1987), providing a unique opportunity to study the structure-function relation of this protein. This thesis has used immunological methods to characterize the structure of the mammalian muscle receptor and to study the structural changes during development that underlie the changes in its functional properties.

Molecular Structure and Functional Properties of the Nicotinic Acetylcholine Receptor

The nicotinic acetylcholine receptor (AChR), a cation-selective ion channel protein, is located in the postsynaptic membranes of vertebrate muscle fibers and of the related cells in electric organs in various fishes. It binds the neurotransmitter acetylcholine released by the nerve terminal and, as a result of the binding reaction, undergoes a conformational change which leads into a

rapid increase and subsequent decrease in the permeability of the endplate membrane to the passage of cations. The inward flux of ions through the ion channels opened by the transmitter is a passive process driven by electrochemical gradients across the receptor-containing membrane. The physiological effect of this influx is a temporal depolarization of the membrane in the synaptic region, leading to muscle contraction in the case of neuromuscular junction, or to an electric discharge in the case of the electric organs.

Early studies on the nicotinic acetylcholine receptor were catalyzed by the discovery of snake venom neurotoxins, such as α-bungarotoxin (α-BuTx) and α-cobratoxin (Chang and Lee, 1963; Lee et al., 1972; Lee, 1979), and by the availability of tissue sources rich in the acetylcholine receptor, the electric organs of *Torpedo* [1-2 nmole/gram of electric organ tissue (Karlin, 1980; Changeux, 1981)] and *Electrophorus*. The neurotoxins bind with very high affinity to the receptor at or near the acetylcholine binding sites of the receptor. α-BuTx, which binds essentially irreversibly to the receptor, and which binds even after receptor has been denatured (Haggerty and Froehner, 1981; Tzartos and Changeux, 1983; Wilson et al., 1984) has been widely used as a radiolabelled probe to quantitate the receptor, to localize it, or to follow its metabolic turnover. α-Cobratoxin, on the other hand, binds with lower affinity, thus providing a major tool for the rapid isolation of large quantities of the receptor from various tissues by affinity chromatography.

More recent advances in the understanding of the functional, biochemical, and biophysical properties of the receptor have come from the development of specific antibodies to the receptor, the availability of molecular cloning techniques and the technology of patch clamping. These tools have greatly advanced our understanding of the molecular structure as well as the functional properties of the nicotinic acetylcholine receptor.

Molecular structure of *Torpedo* AChR. Because the *Torpedo* AChR can be easily obtained in large quantities, its structural and biochemical features have been thoroughly characterized (McCarthy et al., 1986; Popot and Changeux, 1984; Stroud, 1983; Conti-Tronconi and Raftery, 1982, Karlin, 1980). The purified *Torpedo* AChR is a multisubunit integral membrane glycoprotein with an aggregate molecular weight of 250,000 to 275,000 and a sedimentation coefficient of 9S (Weill et al., 1974; Martinez-Carrion et al., 1975; Renolds and Karlin, 1978; Raftery et al., 1980; McCarthy et al., 1986). It contains 4 polypeptides, designated α, β, γ, and δ subunit, respectively, in a molar stoichiometric ratio of $\alpha_2\beta\gamma\delta$ (Renolds and Karlin, 1978; Raftery et al., 1980; Lindstrom et al., 1979, 1980a; Conti-Tronconi and Raftery, 1982). On denaturing gels, these subunits migrate at apparent molecular masses of 40 (α), 50 (β), 60 (γ) and 65 (δ) kDa, respectively (Weill et al., 1974; Lindstrom et al., 1979, 1980a, b; Vandlen et al., 1979; Raftery et al., 1980, 1983). In the electric organs of *Torpedo* and *Electrophorus*, 13S AChR dimers form in the plasma membrane through disulfide bonds between the δ subunits of neighboring receptors (Chang and

Bock, 1977; Hucho et al, 1978; Hamilton et al., 1977, 1979). The genes for the four subunits that constitute the minimal acetylcholine receptor have been cloned and the amino acid sequences deduced (Numa et al., 1983; Patrick et al., 1983).

Reconstitution of the purified AChR into artificial lipid vesicles (McNamee and Ochoa, 1982; Ochoa et al., 1983; Jones et al., 1987) or planar bilayers (Montal et al., 1984; Labarca et al., 1984, 1985) have demonstrated that the four polypeptides (α, β, γ, and δ) alone are sufficient to support both the ligand binding activity of the receptor and the ligand-gated channel activities associated with it *in vivo*. Thus, the receptor and the channel is the same protein.

The primary structure of each subunit deduced from corresponding cDNA sequence has proved uniquely important in providing structural detail and insights into the function of the molecule. The four subunits are highly homologous to each other, with an overall homology of greater than 50% within the four subunits (Noda et al., 1983; Numa et al., 1983, Fairclough et al., 1983), suggesting a similar structural motif for each subunit and a similar contribution of each subunit to the total structure of the receptor. It also suggests that the four subunit genes are derived from a single ancestral gene during evolution.

The deduced amino acid sequences show that each precursor chain contains an N-terminal leader sequence, which is absent in the mature polypeptide (Raftery et al., 1983). Hydrophobicity analysis

reveals four hydrophobic stretches of about 20 or more amino acid residues in length in each polypeptide (Numa et al., 1983; Patrick et al., 1983; Fairclough et al., 1983). These hydrophobic domains have been proposed to be the membrane-spanning regions (M1-M4) (Numa et al., 1983; Patrick et al., 1983; Fairclough et al., 1983). An additional region, the amphipathic region, MA, has also been shown to be conserved among subunits (Finer-Moore and Stroud, 1983). It was proposed that this region also spans the membrane and is responsible for the formation of the ion channel (Finer-Moore and Stroud, 1983, Guy, 1984). More recent studies, however, have provided evidence suggesting that the MA region does not span the membrane (Dwyer, 1988; Roth et al., 1987) and that the ion channel is formed by the M2 region (Imoto et al., 1986; 1988; Tobimatsu et al., 1987; Guy and Hucho, 1987).

Although highly homologous to each other, each subunit is encoded by a separate gene that is located on a different chromosome. In mouse, the α gene is located on chromosome 17, β on chromosome 11, and the γ and δ genes on chromosome 1 (Heidmann et al., 1986).

Each polypeptide of the receptor is N-glycosylated (Vandlen et al., 1979; Anderson and Blobel., 1981; Nomoto et al., 1985; see also Chapter 5). The α and β subunits contains only high-mannose oligosaccharides that are sensitive to endoglycosidase H (endo H), whereas the γ and δ subunits contain both high-mannose and complex types of sugars (Nomoto et al., 1985; see also Chapter 5). AChR

subunits are also subjected to other modifications, including phosphorylation (Vandlen et al., 1979), disulfide formation (Merlie and Lindstrom, 1983), methylation (Kloog et al., 1980; Flynn et al., 1982) and fatty acid acylation (Olson et al., 1984). Some of the these posttranslational modifications have been implicated in the regulation of receptor synthesis and function.

Each subunit polypeptide has its N-terminus located extracellularly, its C-terminus located intracellularly (Lindstrom et al., 1984; Young et al., 1985; but see McCrea et al., 1987) and crosses the lipid bilayer of the membrane several times (Strader et al., 1980; Wennogle and Changeux, 1980; Anderson and Blobel, 1981). Altogether, the 5 polypeptides are arranged in the order $\alpha\beta\alpha\gamma\delta$ (Kubalek et al., 1987) to form a funnel-shaped rosette structure of about 110 Å in its overall length, extending 55 Å beyond the extracelluar surface of the membrane and about 15 Å into the cytoplasm (Ross et al., 1977). The receptor is about 80-90 Å in diameter at the extracellular surface (Klymkowsky and Stroud, 1979). Each rosette contains a stained central pit or channel (Cartaud et al., 1978; Heuser and Salpeter, 1979) of about 25 Å in diameter that can be filled with stain to a depth of about 114 Å (Kistler et al., 1982). This channel has been shown to conduct ions of less than 6.5 Å in diameter at rates comparable to the rate of sodium ion (Maeno et al., 1977, Dwyer et al., 1980).

Role of AChR subunits. Each pentameric AChR molecule contains two toxin-binding sites, corresponding to the ACh binding

sites, which are associated with each of the two α subunits (Conti-Tronconi and Raftery, 1982; Popot and Changeux, 1984; Haggerty and Froehner, 1981; Tzartos and Changeux, 1983; Wilson et al., 1984). Although α-cobratoxin binds equally well to both sites, as does α-BuTx (Sine and Taylor, 1980; Gu et al., 1985; see also Chapter 2), other ligands such as 4-(N-maleimido-benzyl trimethyl ammonium) (MBTA) (Damle and Karlin, 1978; Walker et al., 1984b), bromoacetylcholine (BACh) (Damle et al., 1978; Wolosin et al., 1980; Ratnam et al., 1986; Lyddiatt et al., 1979), d-tubocurarine (Neubig and Cohen, 1979; Sine and Taylor, 1981; Hamilton et al., 1985; see also Chapter 2), carbamylcholine (CARB) (Sine and Taylor, 1980; Dowding and Hall, 1987) and lophotoxin (Culver et al., 1984) bind to one site with higher affinity than to the other. These two sites are also immunologically distinct. Antibodies in some myasthenic sera have been found to distinguish the two sites (Watters and Maelicke, 1983; Whiting et al., 1985; Gu et al., 1985; see also Chapter 2), and monoclonal antibodies have been developed that distinguish the two sites (Fels et al., 1986; Milhovilovic and Richman, 1984,1987; Dowding and Hall, 1987). In one of the most extensive studies (Dowding and Hall, 1987), 10 monoclonal antibodies have been developed that distinguish the two sites, of which 6 are directed to one site and the rest to the other. The functional significance of the pharmacologically and immunologically defined asymmetry between the two sites is unknown. It may be the result of the pentameric structure, which determines that the two α-subunits cannot have the same neighboring subunit chains (Kubalek et al., 1987), or it could arise from structural difference between the two α subunits.

Although the two α subunits are coded for by a single gene (Klarsfeld et al., 1984), they could have different posttranslational modifications. One report has suggested that the two subunits are differentially glycosylated, with one subunit more heavily glycosylated than the other (Conti-Tronconi et al., 1982).

The roles of the subunits other than α subunit are less well defined. Recent molecular and electrophysiological studies of the receptor in developing muscle have indicated that the γ and ε subunits are involved in the developmental change in the channel properties of the receptor (Mishina et al., 1986; Witzemann et al., 1987; Gu and Hall, 1988; see also Chapter 4). The δ subunit has also been suggested to be involved in determining the rate of ion transport through the open channel. Oocytes injected with *Torpedo* α, β, γ, and δ subunit mRNAs express AChRs with a different single channel conductance than those injected with *Torpedo* α, β, γ, and bovine or mouse muscle δ subunit mRNAs (White et al., 1985; Imoto et al., 1986). Detailed analysis of AChRs containing chimaeric δ-subunits and AChRs with single point mutations in each of the four subunits has suggested that clusters of negatively charged and glutamine residues in a region comprising the putative transmembrane segment M2 and the adjacent bend portion between segments M2 and M3 is important for determining single channel conductance (Imoto et al., 1986; 1988).

The γ and δ subunits may also be involved in the regulation of the rate of AChR desensitization. Phosphorylation of the γ and δ

subunits has been shown to increase the rate of receptor desensitization (Albuquerque et al., 1986; Middleton et al., 1986; Huganir et al., 1986; see also Wagoner and Pallotta, 1988). The β subunit, on the other hand, has been proposed to be associated with the 43 KDa protein (Burden et al., 1983), which is an actin-binding protein (Walker et al., 1984a). Thus, it may have a function in receptor localization.

Although the role of each individual subunit is not well understood, it has been demonstrated that all four subunits are labeled by non-competitive channel blocking agents, such as triphenylmethylphosphonium ($TPMP^+$) and chlorpromazine (Hucho et al., 1986; Oberthur et al., 1986; Giraudat et al., 1986,1987), suggesting that the channel is formed by all four subunit types.

Mammalian Muscle AChR Structure. The acetylcholine receptor isolated from mammalian muscle shares many physiological and biochemical properties with the *Torpedo* AChR. AChRs purified from muscles of human, rat , bovine, and mouse muscle all have a subunit structure similar to that of the *Torpedo* receptor (e.g. Nathanson and Hall, 1979; Turnbull et al., 1985; Conti-Tronconi et al., 1982; Brockes and Hall, 1975a; 1975b; Shorr et al., 1981; Lo et al., 1981; Einarson et al., 1982). Furthermore, most antibodies raised against AChRs of *Torpedo* or *Electrophorus* cross-react with muscle receptors (Lindstrom et al., 1979; Tzartos et al., 1981; Sargent et al., 1984; Froehner et al., 1983; Black et al., 1987), suggesting that they share a similar structure. Most importantly, cDNA or genomic

clones corresponding to each of the four subunits of the *Torpedo* receptor have been isolated from mouse (Lapolla et al., 1984; Boulter et al., 1985; Buonanno et al., 1986; Yu et al., 1986;), bovine (Noda et al., 1983; Takai et al., 1984; Tanabe et al., 1984; Kubo et al., 1985), and human (Noda et al., 1983; Shibahara et al., 1985) muscles as well as from chick and *Xenopus* (Nef et al., 1984; Shieh et al., 1987; Baldwin et al., 1988). An additional subunit, ε, which has about 50% homology to the γ subunit has also been isolated from fetal bovine muscle (Takai et al., 1985).

As in *Torpedo* AChR, the amino acid sequences of mammalian muscle AChR are highly homologous between different subunits. And more importantly, corresponding subunits of AChRs from different species also have strong homology, although their sizes vary slightly from one to another. Thus, for example, the human and calf muscle AChR α chains share 97% homology, and over 80% homology exists between human and *Torpedo* α chains (Noda et al., 1983).

The muscle AChR, however, undergoes several changes in their functional properties during the development and maturation of the neuromuscular junction. These changes are discussed below.

Developmental changes of AChR during the formation and maturation of the neuromuscular junction.

The neuromuscular junction is a highly specialized region that occupies less than 0.01% of the total muscle fiber surface. At the

site of contact, both motor nerve terminal and the muscle fiber surface as well as the basal lamina form highly specialized structures. The postjunctional muscle membrane has deep invaginations to form junctional folds (Robertson, 1956; Couteaux, 1958). The most striking specialization at the adult neuromuscular junction in skeletal muscle is the clusters of AChRs organized along the tops of the junctional folds of the postjunctional muscle membrane. They occur in a semi-crystalline array with a packing density of 1 - 2.5 X $10^4/\mu m^2$ (Peper and McMahan, 1972; Anderson and Cohen, 1974; Kuffler and Yoshikami, 1975; Fertuck and Salpeter, 1976; Matthews-Bellinger and Salpeter, 1978). Very low levels ($<100/\mu m^2$) of AChR can be detected outside this region (Fertuck and Salpeter, 1976).

The formation of a mature postjunctional membrane takes several weeks in rats and mice (Salpeter, 1987). During this period, AChRs undergo several sequential changes in their functional properties, including changes in their distribution along the muscle fiber surface, in their metabolic stability and in their channel kinetics (Salpeter and Loring, 1985; Salpeter, 1987; Schuetze and Role, 1987). These changes will be each considered below.

Surface distribution of AChRs during development. During myogenesis, in the early stages of embryonic development, AChRs are distributed more or less evenly over the entire muscle fiber surface at a density of 200-1000 sites/μm^2 (Sytkowski et al., 1973; Bevan and Steinbach, 1977; Burden, 1977a, b; Land et al.,

1977; Dennis et al., 1981; Ziskind-Conhaim et al., 1984; Fischbach et al., 1979; Fambrough, 1979) . Upon nerve-muscle contact, however, receptor clusters begin to be detected underneath the nerve terminal. In rat, nerve-muscle contact is first seen at embryonic day 13, and functional synaptic transmission is established at embryonic day 14, coinciding with myotube formation (Dennis et al., 1981). No postsynaptic specializations are observed at these times. Within 48 hours after nerve-muscle contact, however, AChR clusters appear underneath nerve terminals (Bevan and Steinbach, 1977; Braithwaite and Harris, 1979; Steinbach, 1981; Creazzo and Sohal, 1983). The receptor densities seen at such early nerve-muscle junctions are still very low compared to adult endplates (Bevan and Steinbach, 1977; Matthews-Bellinger and Salpeter, 1983; Ziskind-Conhaim et al., 1984). Moreover, the clusters are unstable and can be dispersed by several agents, such as azide, carbamylcholine, low Ca^{2+} or high K^{+} (Bloch and Steinbach, 1981). Mature, adult type AChR clusters are not seen until 1-3 weeks after birth. At the same time, the density of extrajunctional membrane receptors decreases with development, and by 1 week after birth is almost undetectable (Bevan and Steinbach, 1977).

The mechanisms involved in the initial clustering of the receptor and the maintenance of these clusters in later stages is unknown. Although spontaneous clustering of receptors does occur in some muscle fibers in the absence of nerve, especially in cultured cells *in vitro*, nerve-muscle contact seems essential for initial induction of receptor clusters *in vivo*. Several factors of neuronal

sources have been described that are capable of inducing receptor clusters in cultured myotubes (e.g. Connolly et al., 1982; Buc Caron et al., 1983; Olek et al., 1983; Usdin and Fischbach, 1986).

Earlier experiments have suggested that at least some of the clustered receptors at the site of nerve-muscle contacts initially arise from the redistribution of preexisting AChRs in the extrajunctional membrane (Anderson and Cohen, 1977; Frank and Fischbach, 1979; Ziskind-Conhaim et al., 1984; Role et al., 1985). Basal Lamina seems to be involved in maintaining the cluster. Burden et al. (1979) reported that if the muscle is damaged at the time of denervation, regenerating muscles elaborate postsynaptic specializations, including the AChR cluster at the original synaptic site, even in the absence of the nerve. This observation suggests that some synapse-specific components of basal lamina are involved in maintaining the AChR cluster at the synaptic site. Local expression of AChRs in the synaptic region may also play a role in maintaining the cluster. Northern blot analysis has shown that in normal adult muscle, mRNAs coding for AChR subunits are more abundant in junctional than in extrajunctional regions of the muscle (Merlie and Sane, 1985). More importantly, *in situ* hybridization analysis with AChR probes has shown that in normal adult muscle, AChR mRNA is expressed preferentially in nuclei underneath the motor nerve terminal (Fontaine et al., 1988). Thus, although other mechanisms may be involved, selective expression of AChR in the synaptic region must play at least a partial role in the maintenance of receptor clusters underneath the nerve in adult muscle.

Denervation supersensitivity. In adult innervated muscle, the AChRs are almost exclusively localized at the postsynaptic membrane of the neuromuscular junction (Salpeter, 1987). Very low activity can be detected outside the region. Denervation of the muscle, however, results in an enhanced sensitivity to acetylcholine along the entire muscle surface. This phenomenon, which is the basis of the classical observation of denervation supersensitivity (Cannon and Rosenblueth, 1949; Axelsson and Thesleff, 1959), was first described by Miledi (1960) in frog muscle and was then later found in other species as well.

The basis of the enhanced sensitivity is an increased density of receptors along the muscle fiber surface, due to increased synthesis and insertion of new AChR molecules into the extrajunctional muscle membrane (Brockes and Hall, 1975c; Devreotes and Fambrough, 1976). These extrajunctional receptors have functional properties similar to those of embryonic AChRs. Recent molecular studies have demonstrated that the increase in AChR synthesis in denervated muscle comes from an increase in the level of mRNAs coding for the receptor subunits (Merlie, et al., 1984; Witzemann et al., 1987; Evans et al., 1987; Shieh et al., 1987; Goldman et al., 1985, 1988).

The increase in surface AChR density or mRNA levels in denervated muscle can be prevented or reversed by direct electrical stimulation of the denervated muscle fiber (Linden and Fambrough,

1979; Lomo and Westgaard, 1975; Reiness and Hall, 1977; Goldman et al., 1988). Conversely, inhibition of muscle activity by the sodium channel blocker, tetrodotoxin (TTX), increases the mRNA level and the rate of AChR synthesis (Klarsfeld and Changeux, 1985). These observations strongly suggest that muscle activity suppresses the expression of AChR by reducing the transcription of AChR genes and that denervation supersensitivity is the result of a removal of such a suppression.

Metabolic stability of AChR at the developing endplates. In addition to changes in their distribution, AChRs also change their metabolic stability during development. Receptors in embryonic muscle have a rapid turnover time in the membrane (half-life about 1 day) (Devreotes and Fambrough, 1975; Burden, 1977a, b; Steinbach et al., 1979; Reiness and Weinberg, 1981), whereas receptors at adult neuromuscular junctions turnover with a half life of 8 to 11 days (Burden, 1977a, b; Reiness and Weinberg, 1981; Steinbach et al., 1979; Berg and Hall, 1975; Chang and Huang, 1975; Salpeter and Harris, 1983). In rodents, the degradation rate becomes slower around embryonic day 20, soon after innervation (Reiness and Weinberg, 1981). At roughly the same time, the cluster itself appears to become stabilized and to become more resistant to dispersal by high potassium or energy blocking agents (Bloch and Steinbach, 1981). In chick, the change in turnover time occurs later, about 2 weeks after hatching (Burden, 1977a, b).

Although the mechanisms involved in the metabolic stabilization of the AChR during development are unknown, it is clear that it is not a secondary effect of receptor clustering, as receptor clustering and metabolic stabilization occur at different stages of development. In addition, it has been shown that extrajunctional receptors on embryonic myotubes, whether clustered or diffuse (Devreotes and Fambrough, 1975; Burden, 1977a, b; Steinbach et al., 1979; Reiness and Weinberg, 1981), have a short half life, whereas endplate AChRs in adults are degraded slowly whether they are at the top of the folds, where they are tightly packed, or at the bottoms of the folds, where their density is musch lower (Salpeter and Harris, 1983).

The role of nerve-muscle contact in metabolic stabilization of the receptor has been addressed by Salpeter and colleagues in a series of experiments, in which they followed the metabolic turnover of preexisting endplate receptors after denervation and reinnervation of the muscle (Levitt and Salpeter, 1981; Salpeter et al., 1986). These experiments showed that the turnover of AChRs at adult endplate increases upon denervation and returns to a normal rate following reinnervation. Thus, the nerve seems to play a major role in determining the rate of metabolic turnover of endplate AChRs.

Channel properties of AChR in developing skeletal muscle. The most striking developmental change in the functional properties of the receptor at mammalian neuromuscular junction is the change in

channel properties, which, in rats, occurs during the first and second weeks after birth. A difference in channel properties of muscle AChRs was first detected by Katz and Miledi (1972), who used the technique of noise analysis to study the "elementary current pulse ('shot effects') produced by the action of acetylcholine molecules" in normal and denervated adult frog muscle. They found that endplate channels differ from those that appear in the extrajunctional membrane after denervation both in the "duration of the elementary current pulse" and in the "elementary voltage change". These pioneering results, which have been confirmed and extended by others using techniques such as single channel analysis and analysis of miniature endplate potentials (mepps), showed that adult endplate AChR channels differ from extrajunctional ones in denervated muscle and in embryonic muscle in their single channel conductance (γ) and in their mean channel open time (τ) (Sakmann, 1978; Sakmann and Brenner, 1978; Schuetze and Fischbach, 1978; Fischbach and Schuetze, 1980; Michler and Sakmann, 1980; Vicini and Schuetze, 1985). In rats, embryonic muscles express AChRs with a single channel conductance of about 35 pS and a mean channel open time of about 6 msec at room temperature (slow channel). In contrast, adult endplates express AChRs with a γ of about 50 pS and a τ of about 1 msec (fast channel). The transition from slow to fast channels occurs at developing endplates during the first and second postnatal weeks (Sakmann and Brenner, 1978; Schuetze and Fischbach, 1978; Fischbach and Schuetze, 1980; Vicini and Schuetze, 1985; Witzemann et al., 1987). During this period, both channel types co-exist at the endplate, with the fraction of slow channel

decreasing relative to that of fast channel during the course of development (Vicini and Schuetze, 1985).

Similar changes in AChR channel properties during development have been reported in other species, including human (Cull-Candy et al., 1978; 1982), mouse (Steele and Steinbach 1986; Brehm and Kullberg, 1987; Henderson et al., 1987), and bovine muscles (Mishina et al., 1986) as well in *Xenopus* muscles (Kullberg et al., 1981; Brehm et al., 1982, 1984a, b; Kullberg and Kasprzak, 1985). The only exception reported so far is chick endplate, in which AChR channels express a long τ throughout endplate development (Schuetze, 1980).

The role of the nerve in the transition of AChR channels from slow to fast was addressed in two types of experiments by two independent laboratories. In the first (Schuetze and Vicini, 1984), the transition from slow to fast channel type was examined in rat soleus muscles that had been denervated shortly after birth, when endplates still have mostly slow channels. This experiment established that early denervation blocks or at least attenuates the normal developmental appearance of fast channels. However, if the denervated muscle is stimulated electrically, fast channels still appear (Brenner, 1988), suggesting that the normal transition process during development is a nerve-dependent mechanism that involves nerve-induced muscle activity.

The second type of experiment was to study AChR channel properties in ectopic endplates in denervated adult muscle. AChRs

at new ectopically innervated endplates undergo a similar transition in their channel properties during the second and third weeks after innervation (by a foreign nerve at a site other than the original endplate region) (Brenner and Sakmann, 1978, 1983; Brenner et al., 1987). When the nerve was cut before fast channels appeared, no transition occurred (Brenner et al., 1987). However, if the denervated muscle was stimulated electrically with a chronically implanted electrode after denervation, the AChR at the ectopic endplate acquired fast channels normally (Brenner et al., 1987). Alternatively, if the nerve was maintained intact but inactivated, fast channel also appeared (Brenner et al., 1987). These studies suggest that the nerve exerts its effect both via nerve-induced muscle activity and through nerve-released trophic influences.

Other evidence that the acquisition of fast channels requires innervation comes from studies of embryonic rat myotubes in culture. Siegelbaum et al. (1984) reported that no more than 2-3% of the channel openings were fast in cultured muscles, even in myotubes 2 weeks after plating. Thus, uninnervated muscle does not express AChRs with fast channels to any significant extent.

Although nerve-muscle contact seems to be required for the induction of fast channels *in vivo*, apparently neither nerve nor muscle activity is required for the continuous expression of fast channels at adult endplates. When adult rat muscle are denervated or synaptic transmission blocked presynaptically, at least half of the channels at former endplates are fast ones even 18-20 days

after denervation, when most of the receptors present prior to denervation have presumably been replaced by newly synthesized ones (Brenner and Sakmann, 1983; Sellin and Thesleff, 1981; Levitt and Salpeter, 1981; Salpeter et al., 1986). A similar conclusion is made by Henderson et al. (1987), who have studied the channel properties of newly synthesized AChRs on the surface of denervated muscle by single channel analysis. They found that in both synaptic and non-synaptic regions, both slow and fast channels appear after denervation and that the ratio between the two types of channels is about the same for the two regions. Thus, the change in channel type is independent of the location of AChRs on the muscle surface.

Mechanisms of channel conversion. The phenomenon of channel conversion has been extensively studied both electrophysiologically and biochemically. Based on these studies, several hypotheses have been developed to explain the apparent transition of AChR channels from slow to fast.

The single-AChR hypothesis. The first hypothesis proposed that slow channels and fast ones are the same molecules that are in different membrane microenvironments. Studies of AChRs in reconstituted membranes have established that the ability of the receptor to undergo both agonist-mediated allosteric transitions and channel gating is highly dependent on the lipid environment (Criado et al., 1984; Fong and McNamee, 1986, 1987; McNamee et al., 1986). Thus it is reasonable to assume that changes in the membrane environment that might occur during development may

account for the changes in the channel properties of the receptor. This hypothesis resulted mainly from two electrophysiological observations. The most important observation was made in denervated adult muscle, in which the appearance of AChRs at the nonsynaptic region of the muscle following denervation permits a direct comparison of junctional and extrajunctional receptor with noise analysis. In denervated muscle, noise analysis revealed a clear functional difference between receptors present at the endplate and the newly inserted receptors at the extrajunctional region (Brenner and Sakmann, 1983). These observations prompted the idea that the nerve locally confers adult type channel properties specifically to receptors that are present at the synapse. Further, the observation that this functional distinction persisted for long periods of time after denervation, during which the original receptors would have been expected to be replaced by newly synthesized ones (Brenner et al., 1983; Brenner and Sakmann, 1983), led to the "nerve imprint" hypothesis which proposes that the nerve confers junctional type channel properties on receptors in the synaptic region of the muscle membrane by permanently altering or "imprint" the membrane environment in which the receptor resides (Brenner et al., 1983). Although the nature of the "nerve imprint" was unspecified, it was proposed that such an environmental change would alter or modify the structure of the receptor either directly or by posttranslational modifications.

The hypothesis of channel conversion has also been supported by studies of AChRs in developing muscles. In rat, AChRs change from

slow to fast during the first and second weeks after birth. Comparison of the rate at which apparent channel conversion occurs as detected by electrophysiological measurements, which probably overestimated the proportions of high conductance channels (Brehm, 1988), to the rate of turnover of the receptor has revealed that the apparent channel conversion occurs faster than can be explained by the replacement of surface AChRs with newly synthesized ones (Michler and Sakmann, 1980; Schuetze and Vicini, 1986). Thus, it was proposed that post-translational changes in the structure of the receptor must take place, leading to an alteration in the functional properties of pre-existing receptors.

The two-AChR hypothesis. The single-AChR hypothesis has recently been challenged by evidence from biochemical and molecular studies and from recent electrophysiological studies with patch clamping technology. These studies suggest that the difference in channel properties arises from biochemical differences intrinsic to the receptor and that different channel types represent different receptor molecules.

The idea that the receptors at embryonic and adult endplates are intrinsically different originally came from observations that AChRs at embryonic and adult endplates are immunologically distinct, as detected by antibodies from myasthenic patients. Myasthenic sera typically contain antibodies that bind to embryonic muscle AChRs preferentially (Dwyer et al., 1981; Reiness and Hall, 1981; Hall et al., 1985; O'Malley et al., 1988). In one particular

serum, virtually all the antibodies are of this type (Hall et al., 1985). Immunocytochemical studies showed that these antibodies bind to endplate receptors in neonatal rats but not in adults, and the developmental loss of the binding activity parallels the loss of slow channels at the endplate. Because the antibodies also distinguish AChRs purified from denervated and normal adult rat muscles (Hall et al., 1985), the difference recognized by the antibodies must reside in the receptor itself. Additional evidence that the antibodies specifically recognize AChRs with slow channels came from electrophysiological studies of the effects of the antibodies on AChRs at developing rat endplates, in which both slow and fast type channels exist. It was found that the antibodies blocked the function of receptors with slow channels but not of those with fast channels (Schuetze et al, 1985), demonstrating that the antibodies are specific for AChRs with slow channels. These results, thus demonstrated that AChRs with different channel types are immunologically distinct.

The immunological difference between the two types of receptors could arise either from their being different gene products or from their being differentially modified posttranslationally. Since the first report of the presence of antibodies of such specificities in myasthenic sera, several monoclonal antibodies have been developed that show similar specificities (Souroujon et al., 1985; Whiting et al., 1986). Unfortunately, however, none of these antibodies seemed to recognize denatured protein and thus their subunit specificities and

the basis of the distinction between the two receptor types could not be further characterized.

One line of evidence suggesting that the two types of AChRs result from different gene products has come from studies performed on *Xenopus* myocytes. AChR channels in *Xenopus* myotomal muscle also undergo a change in the single channel conductance and mean channel open time during development (Kullberg et al., 1981; Brehm et al., 1982, 1984a, 1984b; Kullberg and Kasprzak, 1985). The conversion also occurs in myocytes cultured in the absence of nerve (Brehm et al., 1982, 1984b). However, if the cultures were incubated in the presence of cycloheximide, a protein synthesis inhibitor, or tunicamycin, an inhibitor of N-glycosylation that blocks the assembly of newly synthesized AChR subunit polypeptides into intact receptors (Merlie et al., 1982), no channel conversion could be detected (Carlson et al., 1985; Brehm et al., 1987). Treatment of the cultures with the RNA synthesis inhibitors, α-amanitin or actinomycin D, also abolished the appearance of fast channels (Brehm et al., 1987). These results thus demonstrate that new synthesis of protein is required for the channel conversion to occur and that AChRs with different channel types may represent different proteins. These experiments, however, did not prove that new AChR synthesis is required for channel conversion to occur. Treatment with cycloheximide or tunicamycin or with RNA synthesis inhibitors could have inhibited the synthesis or assembly of other proteins that are required to modify the pre-existing receptor. In addition, the channel

conversion observed *in vitro* may not be identical to the conversion *in vivo*.

The first strong evidence suggesting that the AChRs with slow and fast channels are formed from different gene products and that channel conversion *in vivo* is the result of differential gene expression came from the works of Numa and colleagues. In addition to α, β, γ, and δ subunits, Takai et al. (1985) had also cloned a fifth subunit, ε, from fetal bovine muscle. The ε subunit has an overall structure similar to that of other AChR subunits as predicted from the amino acid sequence derived from cDNA sequence and is about 50% homologous to the γ subunit (Takai et al., 1985).

Two sets of experiments were performed by Numa and colleagues that strongly suggested the involvement of the fifth subunit, ε, in the developmental changes in channel properties (Mishina et al., 1986). In the first set of experiments, they injected mRNAs derived from cDNAs encoding various calf muscle AChR subunits into *Xenopus* oocytes and then analyzed the properties of AChR channels that were expressed on the surface with the single channel recording technique. When oocytes were injected with a mixture of mRNAs coding for α, β, γ, and δ subunits, AChRs that were expressed were functionally similar to those of fetal bovine muscle fibers (τ = 13 msec, γ = 40 pS). On the other hand, oocytes injected with a mixture of α, β, ε, and δ subunits expressed AChRs with channel properties similar to those expressed at adult bovine endplate (τ = 6 msec, γ = 60 pS). Oocytes injected with all five

subunit mRNAs (α, β, γ, δ, and ε) expressed both type of channels. These results indicate that AChRs derived from different genes can mimick AChRs of different developmental stages in their channel properties, strongly suggesting that the channel conversion during development *in vivo* is controlled by differential gene expression. This hypothesis is supported by analysis of mRNAs of bovine muscle of different developmental stages. Northern blot analysis showed that γ and ε subunit mRNAs are expressed at different times during development, with γ mRNA expressed at early stages and ε at later stages, corresponding to the expression of slow and fast AChR channels, respectively, at the endplate (Mishina et al., 1986). At intermediate times, during which both types of channels are present, both γ and ε mRNAs are expressed. A similar correlation between the expression of γ and ε mRNAs and the surface AChR channel conversion was also observed in rat muscle (Witzemann et al., 1987).

Although these experiments demonstrated that a switch between γ and ε subunit mimicks the switch of AChR channels from slow to fast, and that the expression of mRNAs coding for these subunits coincides with the expression of the two channel types *in vivo*, these experiments did not prove that the switch from γ to ε is a physiological event. An important piece of evidence that is missing from these studies is to show that the endplates in normal adult muscle express AChRs containing ε, but not γ subunit, whereas the endplates in embryonic muscle express AChRs with γ, but not ε subunit.

More recent electrophysiological studies with the patch clamping technique have also argued against the idea that there is a functional distinction between channels in synaptic and non-synaptic regions. Single channel measurements on adult innervated mouse toe muscle indicated that AChR channels present at the endplate and those of extrajunctional membranes are indistinguishable in their functional properties (Brehm and Kullberg, 1987). In both cases, more than 95% of the channel activities are of the fast type and the rest of the slow type. Thus, it seems that the effect of nerve on AChR channel properties is not restricted to the endplate region, but extends to the entire muscle.

In a similar experiment, Henderson et al. (1987) examined the channel properties of newly synthesized AChRs in junctional and extrajunctional regions of denervated mouse muscles, using single channel analysis. They found, in this case, that greater than 80% of the channel openings were by a low conductance, long open time channel, the remaining being provided by high conductance, brief open time channels. The same proportion of the two channel types was found in junctional and extrajunctional membranes. These results again demonstrated that there is no functional distinction between AChRs in junctional and extrajunctional regions.

Taken together, these studies provided strong evidence that suggests but does not prove that the developmental changes in AChR channel properties in mammalian muscle are due to differential

expression of γ and ε subunits during development. In this thesis (see Chapters 4 and 5), I have developed antibodies that are specific for γ, δ, and ε subunit and have used them to demonstrate that AChRs in embryonic rat muscle contain γ, but not ε subunit, whereas AChRs in normal adult endplates contain ε, but not γ subunit; that AChRs at developing endplates switch from anti-γ immunoreactivity to anti-ε immunoreactivity during the first and second postnatal weeks; and that endplates in denervated muscles contain both anti-γ and anti-ε immunoreactivity.

Other developmental changes. In addition to these major differences between embryonic receptors and mature junctional receptors in distribution, turnover and gating properties described above, differences in response to curare (Beranek and Vyskocil, 1967; Ziskind and Dennis, 1978) and isoelectric point (Brockes and Hall, 1975b; Nathanson and Hall, 1979) have also been reported. In addition to changes in their immunological properties, Hall and colleagues (Brockes and Hall, 1975b; Nathanson and Hall, 1979) reported that AChRs in embryonic endplates and in extrajunctional regions of denervated muscle differ from those of adult innervated endplates in their isoelectric points (pI): AChRs in embryonic muscle and in the extrajunctional region of denervated adult muscle are 0.3 pH units more basic than that of normal adult endplate AChR. This change is already apparent by birth (Brockes and Hall, 1975b).

Clinical Importance of the Acetylcholine Receptor.

The AChRs at the neuromuscular junction are also of clinical interest as they are involved in the disease of myasthenia gravis. Myasthenia gravis is an autoimmune disease in which patients are subject to sporadic weakness and fatigability. The physiological basis of these symptoms is a decreased number of functional AChR in the postsynaptic membranes. Thus, myasthenic endplates show reduced endplate potentials (Elmpuist et al., 1964; Albuquerque et al., 1976a, 1981; Ito et al., 1978; Cull-Candy et al., 1979), a diminished response to iontophoretically applied acetylcholine (ACh) (Albuquerque et al., 1976a; Cull-Candy et al., 1979), and a decreased number of binding sites for α-BuTx (Fambrough et al., 1973; Green et al., 1975).

Antibodies to the AChR in the sera of myasthenic patients (Almon et al., 1974) are thought to be responsible for the loss of junctional AChRs. Antibodies are bound to the postsynaptic membrane of the neuromuscular junctions in myasthenic patients (Engel et al., 1977), and injection of antibodies from myasthenic sera into mice induces symptoms of the disease, with reduced miniature endplate potentials (Mepps) and a decreased number of toxin-binding sites (Toyka et al., 1975).

Different mechanisms are involved in the antibody-mediated reduction in the number of functional receptor (reviewed by Drachman, 1981; Lindstrom and Engel, 1981). In some cases, the antibodies induce a destruction of the postsynaptic membrane by a complement-mediated process. In others, the antibodies decrease

the number of the receptor by increasing its metabolic degradation. In Chapter 3 of this thesis, we report a third mechanism in which the antibodies in the myasthenic serum block the function of the receptor directly.

Chapter 2

The Effects of a Myasthenic Serum on the Acetylcholine Receptors of C2 Myotubes.

I. Immunological Distinction between the Two Toxin-binding Sites of the Receptor

Abstract

We have examined the effect of a serum from a patient with myasthenia gravis on the binding of α-bungarotoxin (α-BuTx) to the acetylcholine receptors (AChRs) of a mouse muscle cell line, C2. After a 2-hr incubation, antibodies in the serum reduced toxin binding to C2 myotubes to a maximal extent of approximately 50%. The degradation of surface AChRs could account for the loss of only 5% of the sites during the incubation; the remainder, therefore, must have been lost by blockage of binding. To investigate whether the antibodies blocked specifically one of the two toxin-binding sites that each AChR possesses, we used an analysis based on that of Sine, S. W., and P. Taylor, ((1981) J. Biol. Chem. 255: 10144-10156). Although the two sites could not be distinguished by their rates of binding of α-BuTx, *d*-tubocurarine (dTC) inhibition of the initial rate of toxin binding revealed that the two sites had affinities for dTC that differed by approximately 30-fold. Incubation with the myasthenic antibodies reduced the number of high-affinity dTC sites, without affecting those of low affinity. We conclude that the two toxin-binding sites of the AChR are immunologically distinct.

Introduction

The antibodies to the acetylcholine receptor (AChR) that are found in the sera of patients with myasthenia gravis are thought to play a crucial role in the pathogenesis of the disease (Vincent, 1980; Drachman, 1981; Lindstrom and Engel, 1981). Thus, muscle weakness and other symptoms of myasthenia can be produced in experimental animals by active immunization against the purified AChR (Patrick and Lindstrom, 1973) or by passive immunization with immunoglobulin from myasthenic patients (Toyka et al., 1975). The antibodies to the AChR in myasthenic sera are heterogeneous, and the poor correlation between their titer and the severity of the disease (Lindstrom et al., 1976; Vincent and Newsom-Davis, 1980; Roses et al., 1981) suggests that only certain classes of them are deleterious. One class in which the concentration correlates with the clinical state of the disease is that of the antibodies that inhibit the binding of α-bungarotoxin (α-BuTx) to the AChR (Drachman et al., 1982).

In addition to their importance in the pathophysiology of the disease, myasthenic antibodies can also serve as useful tools for investigating the structure and regulation of the AChR. Sera from most patients, for example, have antibodies that distinguish the AChRs in the extrajunctional membranes of developing or denervated mammalian muscle from those at mature endplates (Almon et al., 1974; Bender et al., 1975; Weinberg and Hall, 1979; Dwyer et al., 1981; Lefvert, 1982). Although the AChR is extremely well

characterized biochemically, the specificities of myasthenic antibodies have been defined at a molecular level only in a few instances (Tzartos et al., 1982), and their use as structural probes of the AChR has been limited.

We have recently described a myasthenic serum with an unusually high degree of specificity for AChRs in the extrajunctional membranes of developing and denervated muscle. The antibodies in this serum also bind AChRs at developing endplates in rat muscle until the third postnatal week (Hall et al., 1985). The specificity of these antibodies is even more striking in that they appear to bind almost exclusively at or near the toxin-binding sites (Hall et al., 1985). The AChR has two toxin-binding sites, which are associated with each of the two α-subunits of the AChR (Karlin, 1980; Haggerty and Froehner, 1981; Conti-Tronconi and Raftery, 1982; Oblas et al., 1983; Gershoni et al., 1983). In muscle cells, these two sites can be distinguished by agonists and antagonists of the AChR, although not by the toxin itself (Sine and Taylor, 1979, 1980, 1981). We report here experiments in which we have used a mouse muscle cells line, C2, to investigate the specificity of the myasthenic serum for the two toxin-binding sites of the AChR *in situ*. In the following chapter, we examine the effects of the serum on the functional response of C2 AChR to agonists.

Materials and Methods

Myasthenic antibodies. The antibodies have been described previously (Hall et al., 1983, 1985). They are derived from a serum taken at plasmapheresis by Dr. Peter Dau at Children's Hospital in San Francisco in February 1978. A 40% ammonium sulphate precipitate, after storage for 3 years at Children's Hospital at -80 °C, was dialyzed against 0.02 M ammonium bicarbonate, lyophilized, and stored at -20 °C. In all experiments except the immunofluorescence experiment, the source of the antibodies was the lyophilized protein, taken up just before use in Buffer A.

Other reagents. Monoiodinated ^{125}I-α-BuTx was prepared and purified as previously described (Inestrosa et al., 1983). Rhodamine-conjugated α-BuTx was prepared as described by Ravdin and Axelrod (1977). d-Tubocurarine (dTC) was from Calbiochem. An antiserum to the myasthenic antibodies was raised in rabbits using a DEAE fraction of the myasthenic antibody preparation prepared as described by Hall et al. (1985).

Tissue culture. The C2 mouse muscle cell line, originally isolated by Yaffe and Saxel (1977), was maintained as described by Inestrosa et al. (1983). Myoblasts were grown in a medium consisting of Delbeco's modified Eagle's medium with 1 gm/liter of glucose (DME H-16) (Gibco), supplemented with 20% fetal calf serum and 0.5% chick embryo extract (growth medium). To stimulate cell differentiation, growth medium was replaced by DME H-16 supplemented with 10% horse serum (fusion medium). Within

several days, large multinucleated myotubes appeared with AChRs on their surfaces (Inestrosa et al., 1983).

Except where noted, myoblasts were plated in 24-well plastic tissue culture plates (Falcon) at a density of about 3000 cells/cm^2. For measurement of AChR turnover, the wells were precoated with collagen to increase the attachment of cells to the dish. For immunohistochemical staining, the myoblasts were plated on plastic coverslips in 24-well plates. In all cases the growth medium was replaced by fusion medium after 48 hours and the cultures were used for experiments within the next 4 days.

Kinetics of α-BuTx binding in normal and antibody-treated cells. The general procedures for studying α-BuTx binding to C2 cells was similar to that described by Sine and Taylor (1979) for BC3H-1 cells, a non-fusing mouse muscle cell line. Fusion medium was removed from C2 myotubes, and the cells were equilibrated for 20 minutes at room temperature with a buffer consisting of 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.7 mm $MgCl_2$, 1.0 mM Na_2HPO_4, 5.5 mM glucose, 25 mM HEPES, and 60 μg/ml bovine serum albumin, pH 7.4 (Buffer A). The binding reaction was initiated by replacing the medium with the same medium containing 10 nM ^{125}I-α-BuTx. The reaction was terminated at various intervals by aspiration, followed by three 1-ml washes with Buffer A. Cells were then extracted twice in 0.5 ml of 0.1 M NaOH, and the combined extracts were counted in a Beckman II gamma counter. The total number of α-BuTx-binding sites was determined by incubating the cells with

^{125}I-α-BuTx for 3 hours under the same conditions. The association rate constant, k_t, was calculated using the integrated form of the second-order rate equation,

$$\frac{1}{T_0 - R_0} \ln \frac{R_0\, T}{T_0\, R} = k_t\, t$$

Where T_0 is the initial concentration of ^{125}I-α-BuTx, R_0 is the initial concentration of receptor, expressed in α-BuTx-binding sites, and R and T are the concentration of AChR and ^{125}I-α-BuTx, respectively, at time t. R and T were determined by subtracting the amount of toxin-receptor complex present at time t from the initial concentrations, R_0 and T_0, respectively. For experiments with antibodies, the cells were incubated with 4 mg/ml of the antibody in Buffer A for 2 hours at 37 °C before measuring the kinetics of α-BuTx binding. In all experiments ^{125}I-α-BuTx binding in the presence of 100-fold excess of unlabeled α-BuTx was subtracted from the total binding.

Effects of dTC on α-BuTx binding rate. The effects of dTC on the initial rate of α-BuTx binding was monitored with or without pretreatment of the cells with the antibody. After incubation with 4 mg/ml antibody or buffer for 2 hours at 37 °C, cells were rinsed as above and preincubated with various concentrations of dTC or buffer for 20 minutes at room temperature. The medium was then replaced by the same medium containing 10 nM 125I-α-BuTx. After 10 minutes the binding reaction was stopped, the cells washed and the

amount of bound toxin determined as described above. Control experiments established that the formation of toxin-receptor complex was linear with time during this period (see Figure 2-6). The inhibition data were fit to a two-site model for antagonist binding as described by Sine and Taylor (1981):

$$\frac{k_{obs}}{k_t} = N_A \frac{K_A}{K_A + L} + N_B \frac{K_B}{K_B + L}$$

Where k_{obs} and k_t are the association rate constants for α-BuTx binding to the AChR in the presence and absence, respectively, of dTC at concentration L, and N_A and N_B are the relative fractions of binding sites for dTC with dissociation constants of K_A and K_B, respectively. In the absence of antibody, N_A and N_B were both assumed to be equal to 0.5, and the values for K_A and K_B were obtained by linear regression, treating the two extreme portions of the curve separately. Because the difference between K_A and K_B was so large, the equation could be simplified to linear equations at the two extremes of the curve. In experiments involving antibody treatment, data were analyzed by assuming that K_A and K_B were unchanged. The values for N_A and N_B were calculated from the data for total toxin binding inhibition according to different models for the binding of antibody (see results).

AChR degradation. C2 myotubes grown in collagen-coated 24-well plates were rinsed and incubated with buffer or antibody (4-6 mg/ml) for 2 hours. The receptor was then labeled with ^{125}I-α-

BuTx by incubating with 10 nM toxin for 2 hours. After extensive washing to remove free toxin, the myotubes were incubated in 0.5 ml of fusion medium at 37 °C. At intervals of 1 to 3 hours the culture medium was collected, counted and then frozen for later analysis. The cells were re-fed with 0.5 ml of fresh medium. At the end of the experiment, the cells were extracted twice with 0.5 ml of 0.1 M NaOH and the combined extracts were counted. The radioactivity released into the medium at each time point was analyzed by P-2 column chromatography to separate degradation products from intact toxin that might have been released by dissociation of toxin-receptor complex. The data for degradation and dissociation were then plotted separately on a semilog plot as a percentage of total toxin bound. Because both degradation and dissociation affect the same population of toxin-receptor complex, this method of graphical analysis is not rigorously correct; however, it does allow a comparison between cells incubated with and without antibody that is sufficiently accurate for the purpose of the analysis.

Immunofluorescence. Because of the high background found with the crude antibody preparation used in other experiments, immunofluorescence experiments were performed with an immunoglobulin fraction purified by passage over a DEAE column and, in some cases, by further fractionation on a Sephacyl S-200 column. Details of the preparations are given in Hall et al (1985) and in Silberstein et al. (1983). Similar results were obtained with both preparations of the antibody. Myotubes grown on coverslips were incubated with 2 mg/ml of antibody in a total volume of 0.2 ml for

60 minutes followed by fluorescein-conjugated goat anti-human IgG Fab fragment (Cappel Laboratories) and rhodamine-conjugated α-BuTx. In some experiments, the rhodamine-conjugated α-BuTx was added before the myasthenic antibodies.

Results

Binding of myasthenic antibodies to C2 myotubes. Binding of the myasthenic antibodies to C2 myotubes was initially tested by immunofluorescence. Incubation of the myotubes with the myasthenic antibodies, followed by fluorescein-conjugated second antibody and rhodamine-conjugated α-BuTx revealed clusters of AChRs on the myotube surface (Figure 2-1, A and B). As with the AChRs at developing endplates (Hall et al., 1985), preincubation of the myotubes with rhodamine-toxin before incubation with the antibodies completely blocked antibody binding (Figure 2-1, C and D). These results suggested that the antibodies bind at or near the α-BuTx-binding site of the C2 AChR.

This interpretation was confirmed by examining the effects of antibody on the binding of ^{125}I-α-BuTx to the AChRs in C2 myotubes. Preincubation of the cells with increasing concentrations of antibody progressively reduced the binding of ^{125}I-α-BuTx to the myotubes (Figure 2-2). The maximal extent of inhibition seen in several experiments was approximately 50%. This result is similar to those seen earlier with solubilized AChRs from denervated rat muscle or from *Torpedo* electric organ (Hall et al., 1983, 1985).

Effects of antibodies on AChR degradation. Because these experiments were carried out on AChRs in intact cells, a possible explanation of decrease in toxin binding caused by the antibodies is that they increased the rate of internalization and degradation of the surface AChRs. To test this possibility, we incubated the myotubes with the myasthenic antibodies, followed by ^{125}I-α-BuTx to label the AChRs, and then followed the rate of receptor degradation by the appearance of radioactivity in the medium. P-2 column chromatography was used to separate degradation products from intact toxin released by dissociation. The results indicated (Figure 2-3) that, although the antibodies had little or no effect on dissociation of toxin-receptor complex, the rate of degradation of the complex was slightly increased. Thus, the half-time of degradation of surface AChRs measured in the absence of antibody was 13 hours, in agreement with previous results on C2 myotubes (Miller, 1984). Preincubation with the myasthenic antibodies resulted in a decrease in the half-time of degradation to 10.4 hours (Figure 2-3). No further increase in the degradation rate of AChR in myasthenic antibody-treated myotubes was seen upon addition of a second antibody. In some experiments toxin was added before the myasthenic antibodies. In these cases, the antibody also caused an increase in degradation that was similar to that seen when antibody was added before toxin (data not shown).

Since, in our experiments on toxin binding, cells were incubated for 2 hours or less after antibody treatment, the small increase in

AChR degradation could contribute only slightly to the reduction in toxin binding seen in antibody-treated cells. Thus , the increased degradation caused by the antibody in a 2-hour period in the experiment shown in Figure 2-3 resulted in a loss of approximately 5% of the total toxin-binding sites, whereas in this same experiment there was a reduction of 42% in total toxin binding during this period. We conclude that the decrease in number of active toxin-binding sites is largely due to inhibition of toxin binding and not to loss of AChRs from the myotube surface.

The antibodies bind to one of the two toxin-binding sites. Each AChR has two toxin-binding sites associated with each of the two α-subunits that each receptor possesses. One possible explanation for the partial inhibition of toxin binding by the myasthenic antibodies is that the antibodies bind to only one of the two toxin-binding sites on each AChR. This could occur either because the antibodies recognize specifically one of the two sites, or because non-selective binding of antibody to either of the two sites blocks binding to the other. Sine and Taylor (1979, 1980, 1981) have carried out an extensive study of the toxin-binding sites of the AChRs in BC3H-1 cells, a non-fusing mouse muscle cell line. We have used an analysis similar to theirs to investigate the specificity of the binding of the myasthenic antibodies to the AChR of C2 myotubes.

When the binding of α-BuTx to C2 myotubes was measured, kinetic analysis showed a single association rate constant, K_t, of

2.6×10^4 M^{-1} S^{-1} (Figure 2-4). Thus, as in BC3H-1 cells, the two toxin-binding sites have identical or closely similar association rate constants for toxin binding. We also examine the kinetics of toxin binding after incubation with antibody (Figure 2-5). The sites remain after antibody treatment showed an association rate constant of 1.5×10^4 M^{-1} S^{-1}, a value that is about 40% lower than that seen in control myotubes. The decrease could result from reduced accessibility of the remaining binding sites to toxin or to a change in conformation of the AChR induced by antibody binding.

The initial rate of binding of ^{125}I- α-BuTx to the AChR was also decreased by dTC (Figure 2-6). The inhibition occurred over a broad range of dTC concentrations, extending from 10^{-8} M to 10^{-5} M (Figure 2-7). Analysis of the inhibition curve showed that the data were well fit by assuming that there are two independent sites, equal in number, with dissociation constants of $K_A = 5.6 \times 10^{-8}$ and $K_B = 1.6 \times 10^{-6}$ M (Figure 2-7). The data for dTC inhibition could also be explained by negative cooperative interactions between two kinetically identical sites. According to such a model, the two sites would initially have indistinguishable affinities for dTC, but binding of one site would reduce the affinity of the other for dTC. This possibility was examined by blocking a fraction of the binding sites with unlabeled α-BuTx; dTC inhibition of ^{125}I-α-BuTx binding to the remaining sites was then determined. In two experiments in which 75% and 48% of the sites were blocked, the inhibition curves that were obtained (Figure 2-8) were similar to that seen in the absence

of unlabeled toxin. These results are consistent with the interpretation that there are two independent sites.

We then examined the effects of antibody on the inhibition by dTC. In the experiment shown in Figure 2-9, the added antibody reduced the total number of toxin-binding sites by 40%. This decrease could have come about in three ways: (a) selective loss of high affinity dTC sites; (b) selective loss of the low affinity dTC sites; or (c) the loss of equal numbers of both sites. Theoretical curves for each of these possibilities were made, assuming that the antibody causes no change in either K_A or K_B, the dissociation constants for the two sites. The data were best fit by the model in which the antibody caused selective loss of the high affinity site. We therefore conclude that the antibodies in the myasthenic serum selectively block toxin binding to one of the two toxin-binding sites on the AChR.

Discussion

The nicotinic AChR has two sites that bind agonists, antagonists, and elapid neurotoxins. Presumably these are structurally similar sites, since they are associated with the two α-subunits that the receptor contains (reviewed in Karlin, 1980; Conti-Tronconi and Raftery, 1982; Changeux et al., 1984). Experiments with affinity-alkylating agents (Damle and Karlin, 1978; Wolosin et al., 1980) and direct binding studies (Neubig and Cohen, 1979; Fels et al., 1982) with agonists and antagonists have

established that, in *Torpedo* AChRs, the two sites are nonidentical. An extensive and elegant analysis of the two sites of the muscle AChR has been carried by Sine and Taylor (1979, 1980, 1981), using BC3H-1, a non-fusing mouse muscle cell line. They found that the AChRs in BC3H-1 cells bind α-BuTx with a single rate constant, but that inhibition of toxin binding by antagonists reveals two populations of toxin-binding sites. The two populations of sites are of equal concentration and appear to be intrinsically different. The two sites must be associated with the same receptor, because occupation of both sites by agonists was required for activation of the ion channel.

We have used the studies of Sine and Taylor (1979, 1980 1981) as a framework within which to investigate the effects of a myasthenic serum on the AChR of another mouse muscle cell line, C2. This serum is of interest in part because of its biological specificity: it detects a developmental change in endplate AChRs that appears to be related to the channel properties of the receptor (Hall et al., 1985; Schuetze et al., 1985). In C2 cells, the antibodies in this serum reduce toxin binding to the myotubes by about 50%. Although the antibodies cause a slight increase in the AChR degradation rate, this effects is not large enough to account for the observed decrease in toxin binding, which therefore must occur by direct block. The observation that antibody binding to clustered AChR, which can be visualized immunocytochemically, is largely blocked by preincubation with toxin (Figure 2-1) is consistent with this interpretation. This result suggests that most of the antibodies

in this serum are directed toward the toxin binding sites; other antibodies must be present, however, because the serum increases the degradation of preformed toxin-receptor complex on the cell surface (data not shown). The exact proportions of antibodies directed against the toxin-binding site and those directed elsewhere are difficult to estimate from experiments such as these but could be determined by an assay that measured binding directly. We have been unable to successfully establish such an assay, presumably because the low titer of the AChR-specific antibodies and the fact that they appear to be partially degraded (Silberstein et al., 1983).

Our experiments characterizing the toxin-binding sites on C2 myotubes confirm the earlier experiments on BC3H-1. Thus, there is a single binding constant for α-BuTx, but binding of dTC, measured by inhibition of toxin binding, occurred with two dissociation constants. Because the two dissociation constants are unchanged after partial block with toxin, we conclude that the two sites do not arise by negative cooperativity but are intrinsically different. We then used the differential sensitivity of the two sites to dTC to examine the specificity of the myasthenic serum. Our results showed clearly that incubation of the myotubes with the myasthenic antibodies selectively blocked binding of α-BuTx to the site with high affinity for dTC. Thus, the two sites are not only pharmacologically, but also immunologically dissimilar. Evidence is presented in the following chapter that both sites are associated with the same receptor.

There are several possible biochemical sources for the immunological difference between the two sites. Because the subunits in the AChR are arranged asymmetrically, the two α-chains have different neighbors that could differentially alter the conformation of each active site. Alternatively, the difference might be intrinsic to the α subunit. Although current evidence indicates that there is only a single structural gene for the α chain (Noda et al., 1983; Klarsfeld et al., 1984) and that the two α subunits have identical amino acid sequences over the portions of the chain that have been analyzed (Raftery et al., 1980; Conti-Tronconi et al., 1982, 1984), differential transcription or processing could give rise to small differences in the peptide backbone. Post-translational modifications that distinguish the two α-chains could also be the source of the different reactivity to the myasthenic antibodies. The cDNA sequence of the α-subunit indicates that there is a single site of N-linked glycosylation that is adjacent to the active site (Noda et al., 1982; Sumikawa et al., 1982; Devillers-Thiery et al., 1983), and recent biochemical experiments suggest that the two α-subunits may differ in the extent of their glycosylation (Conti-Tronconi et al., 1984).

Antibodies in myasthenic sera that block toxin binding have been described previously and appear to be relatively common (reviewed in Vincent, 1980). In several cases, inhibition to approximately 50% has been observed, suggesting a specificity for one of the two sites as was seen here (Almon and Appel, 1975; Albuquerque et al., 1976; Lefvert and Bergstrom, 1977; Massa and

Mittag, 1983). Further experiments will be required to determine whether other myasthenic antibodies are specific for the site with low affinity for dTC. Antibodies that block toxin binding are of particular clinical interest because their titer in myasthenic sera is correlated with the severity of the disease symptoms (Drachman et al., 1982). As binding to both agonist sites is required for full channel opening, block of one of these sites by antibody might be expected to have functional consequences. The effect of the antibodies described here on the physiological response of the AChR to agonists in C2 myotubes is described in the following chapter.

Figure 2-1. Binding of the antibodies to AChRs in C2 myotubes *in situ*. C2 myotubes grown on coverslips were incubated with an S-200 fraction of the myasthenic serum prepared as described under "Materials and Methods," followed by fluorescein-conjugated second antibody and rhodamine-labeled α-BuTx. Cells were then photographed with fluorescein (*B* and *D*) or rhodamine (*A* and *C*) optics. In *C* and *D* the cells were incubated with rhodamine-labeled α-BuTx before the antibody.

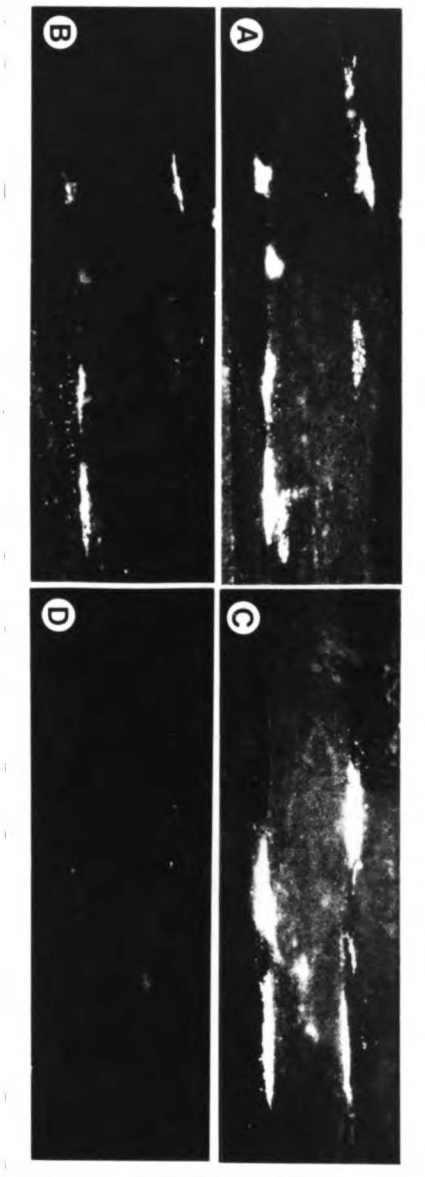

B
A
D
C

Figure 2-2. Myasthenic antibody inhibit toxin binding to AChRs in C2 myotubes *in situ*. C2 myotubes were grown in 24-well plates and were incubated for 2 hours at 37 °C in 0.2 ml of the myasthenic antibodies at the indicated concentrations. Binding of ^{125}I-α-BuTx was then measured as described under "Materials and Methods." Determinations were made in duplicate. The data were the average of the two determinations and were expressed as percentages of the binding in the absence of antibody. In this experiment, the 100% value was 14 fmol/well. *Ab*, antibody.

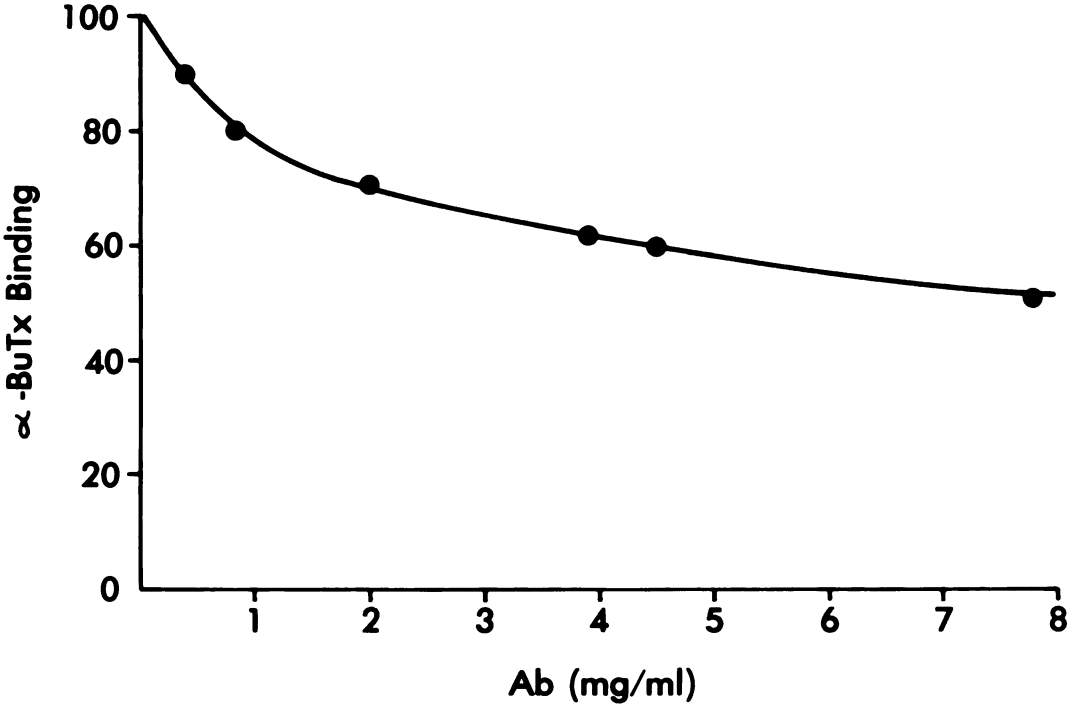

100
80
60
40
20
0
α-BuTx Binding
1
2
3
4
5
6
7
8
Ab (mg/ml)

Figure 2-3. Effect of the antibodies on C2 AChR turnover. C2 myotubes grown in collagen-coated 24-well plates were incubated with 4 mg/ml of the antibody or buffer for 2 hours at 37 °C. The receptors were then labeled with ^{125}I-α-BuTx. The radioactivity released into the culture medium was monitored and passed over a P-2 column as described under "Materials and Methods." Determinations were made in duplicate. In this experiment, the 100% value in the absence of antibody (*-Ab*) was 89.1 ± 4.0 fmol/well (n = 4), and the 100% value in the presence of antibody (*+Ab*) was 51.6 ± 4.5 fmol/well (n = 6). The half-lives ($t_{1/2}$) of the AChR in the presence and absence of antibody were 10.4 and 13 hours, respectively.

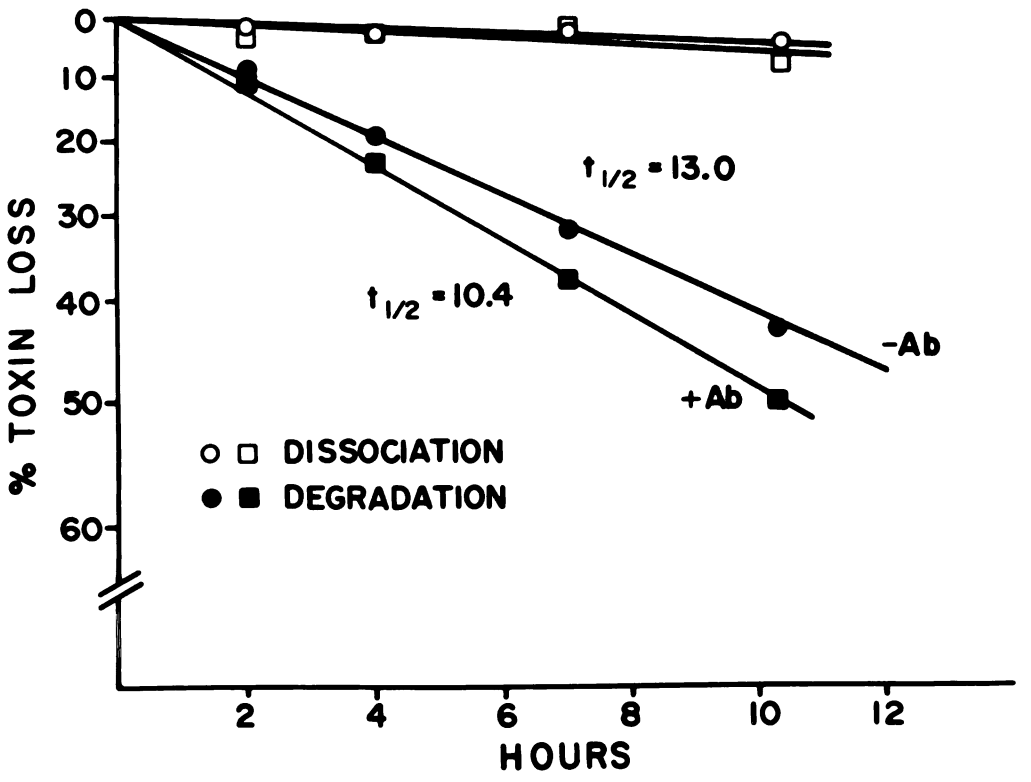

0
10
20
30
40
50
60
% TOXIN LOSS
$t_{1/2}$ = 13.0
$t_{1/2}$ = 10.4
-Ab
+Ab
DISSOCIATION
DEGRADATION
2
4
6
8
10
12
HOURS

Figure 2-4. Kinetics of ^{125}I-α-BuTx binding to C2 AChRs *in situ.* C2 myotubes in 24-well plates were incubated with 10 nM ^{125}I-α-BuTx for various times at room temperature. The binding kinetics were then analyzed using a bimolecular rate equation as described under "Materials and Methods." Linear regression of these data reveals a slope, k_t, of 2.6 x 10^4 $M^{-1}S^{-1}$ (r = 0.992) (*inset*). In this experiment, the total receptor concentration R_0 = 79.4 fmol/well. All *points* were the average of three determinations.

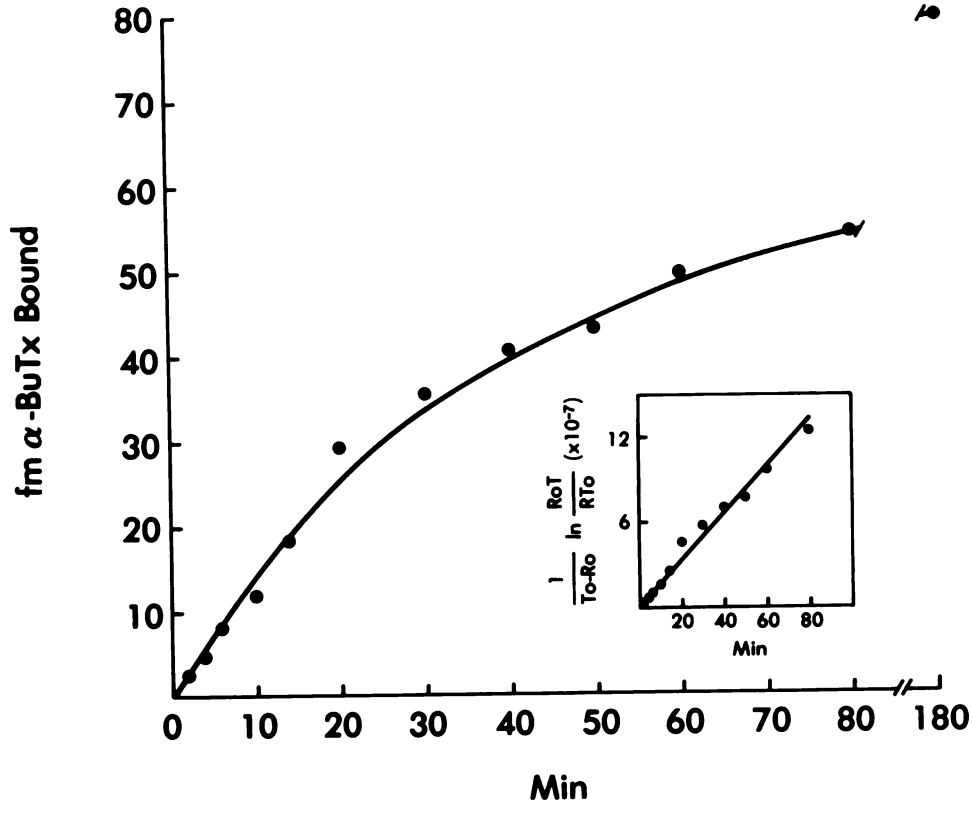

fm α-BuTx Bound
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
180
Min
1/(To-Ro) ln RoT/RTo (x10⁻⁷)
12
6
20
40
60
80
Min

Figure 2-5. Effect of the antibodies on the association rate of ^{125}I-α-BuTx and C2 AChRs *in situ*. The experiment was carried out as described in the legend to Figure 2-4, except that the cells were incubated with a 4 mg/ml of antibody or buffer for 2 hours at 37 °C prior to the initiation of ^{125}I-α-BuTx binding. In the experiment shown, R_0 = 71.2 fmol/well in the absence of the antibody (- *Ab*) and 43.4 fmol/well in the presence of antibody (+ *Ab*). Each *point* is the average of two determinations. In the presence and absence of antibody, the association rate constants were 1.5 x 10^4 and 2.4 x 10^4 $M^{-1}S^{-1}$, respectively.

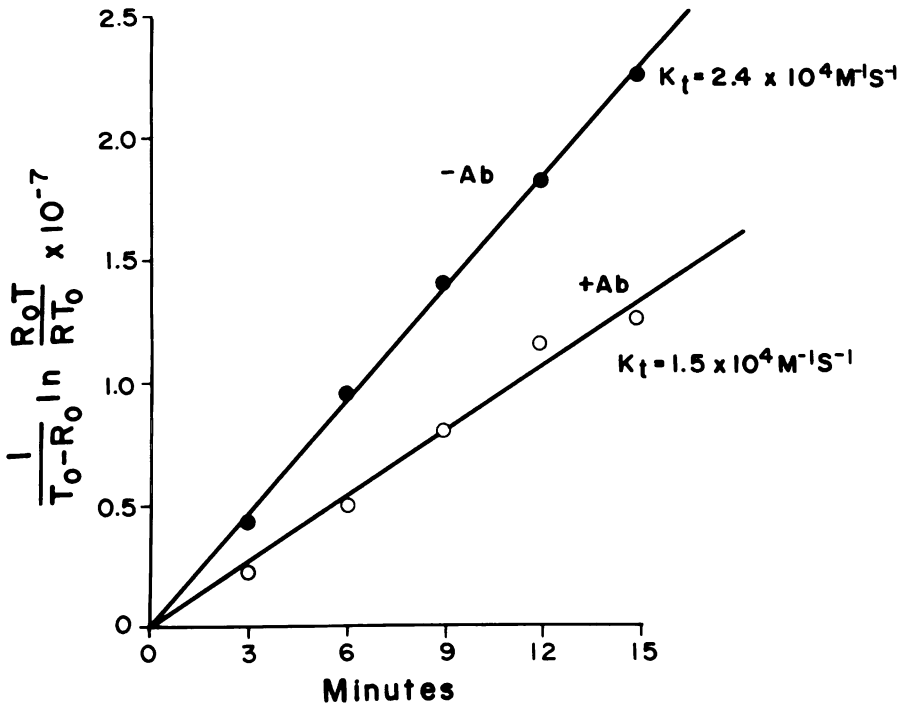

2.5
2.0
1.5
1.0
0.5
0
0
3
6
9
12
15
Minutes
$\frac{1}{T_o - R_o} \ln \frac{R_o T}{R T_o} \times 10^{-7}$
$K_t = 2.4 \times 10^4 M^{-1} S^{-1}$
-Ab
+Ab
$K_t = 1.5 \times 10^4 M^{-1} S^{-1}$

Figure 2-6. ^{125}I-α-BuTx/AChR complex formation in the presence and absence of dTC. C2 cells were rinsed and preincubated with different concentrations of dTC or buffer for 20 minutes at room temperature. The solution was then replaced by the same solution containing 10 nM ^{125}I-α-BuTx. ^{125}I-α-BuTx-AChR complex formation was monitored at different time points. This experiment demonstrated that the ^{125}I-α-BuTx-AChR complex formation was linear for at least 10 minutes under the conditions used.

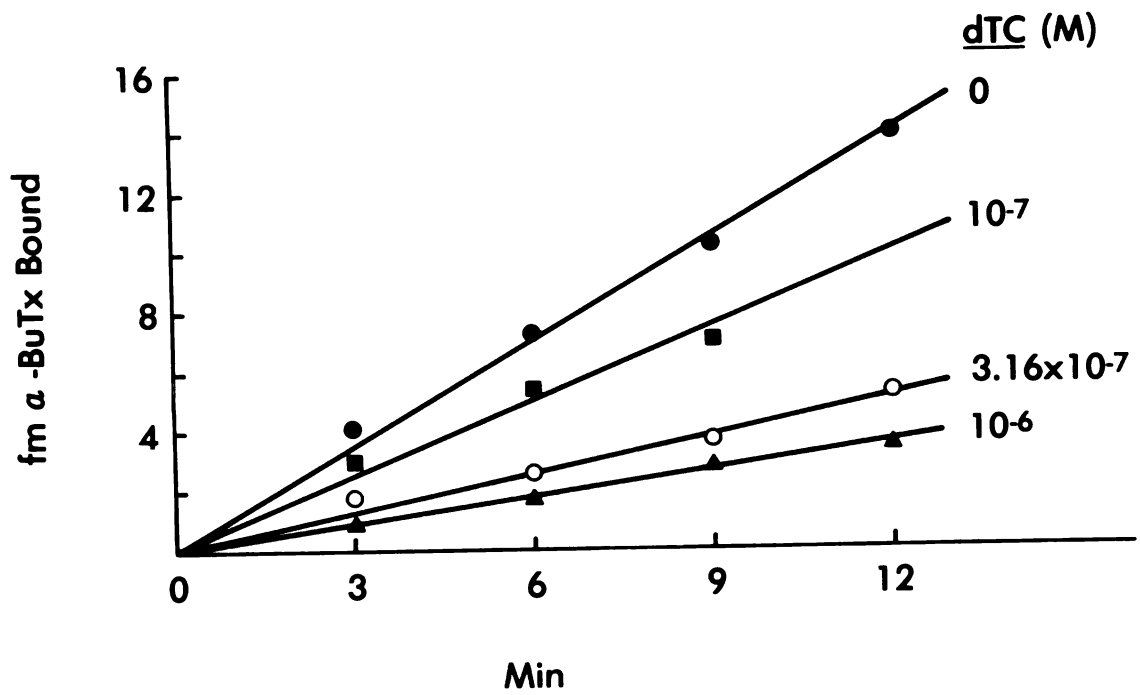

dTC (M)
0
10-7
3.16x10-7
10-6
16
12
8
4
0
3
6
9
12
fm α -BuTx Bound
Min

Figure 2-7. dTC inhibition of the initial rate of ^{125}I-α-BuTx binding to AChRs on C2 myotubes. C2 myotubes in 24-well plates were preincubated at room temperature for 20 minutes with the indicated concentrations of dTC, followed by the same solution containing 10 nM ^{125}I-α-BuTx. The amount of bound toxin in 10 minutes was then determined as described under "Materials and Methods." The data shown were taken from five independent experiments. All individual values were in duplicate and each *point* represents the mean ± SEM. The *solid line* is a theoretical curve expressing inhibition by two independent sites of equal concentration, of which the dissociation constants for dTC are 5.6×10^{-8} and 1.6×10^{-6} M, respectively.

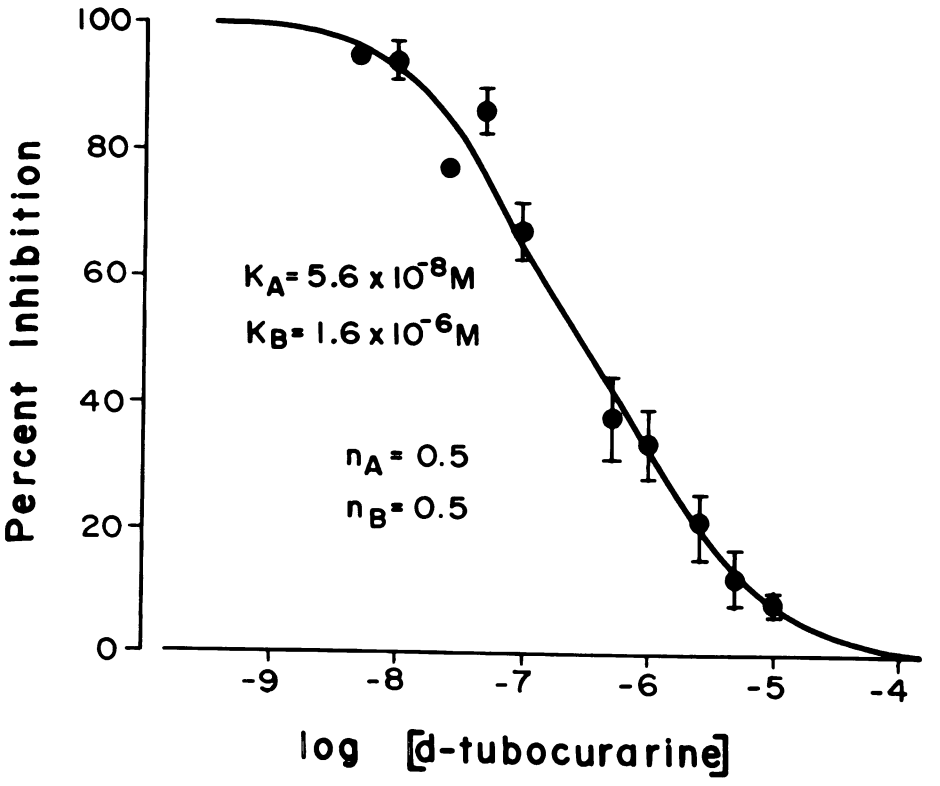

100
80
60
40
20
0
Percent Inhibition
$K_A = 5.6 \times 10^{-8} M$
$K_B = 1.6 \times 10^{-6} M$
$n_A = 0.5$
$n_B = 0.5$
-9
-8
-7
-6
-5
-4
log [d-tubocurarine]

Figure 2-8. Effect of partial block of α-BuTx-binding sites on N_A/N_B ratio. C2 myotubes were incubated with 2.5 nM unlabeled α-BuTx for 1.5 to 2 hours at 37 °C. After extensive washing, the cells were incubated with dTC and then with ^{125}I-α-BuTx as described in the legend to Figure 2-7. The data were from two independent experiments in which the number of active sites was reduced by unlabeled α-BuTx to 52% and 25%, respectively. The *solid line* is the same curve that is shown in Figure 2-7.

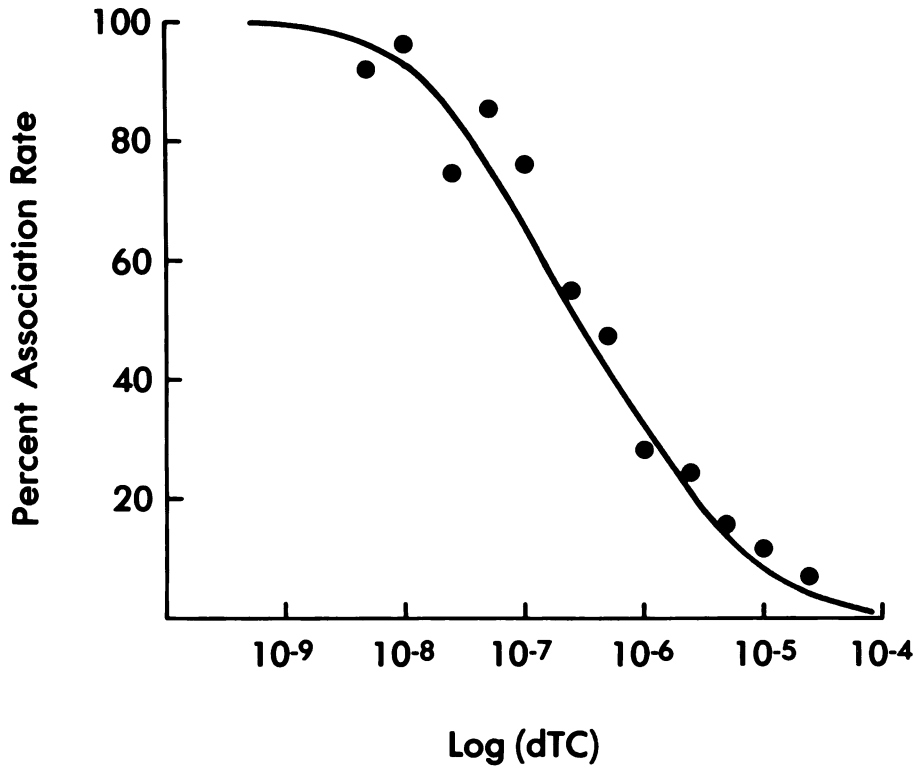
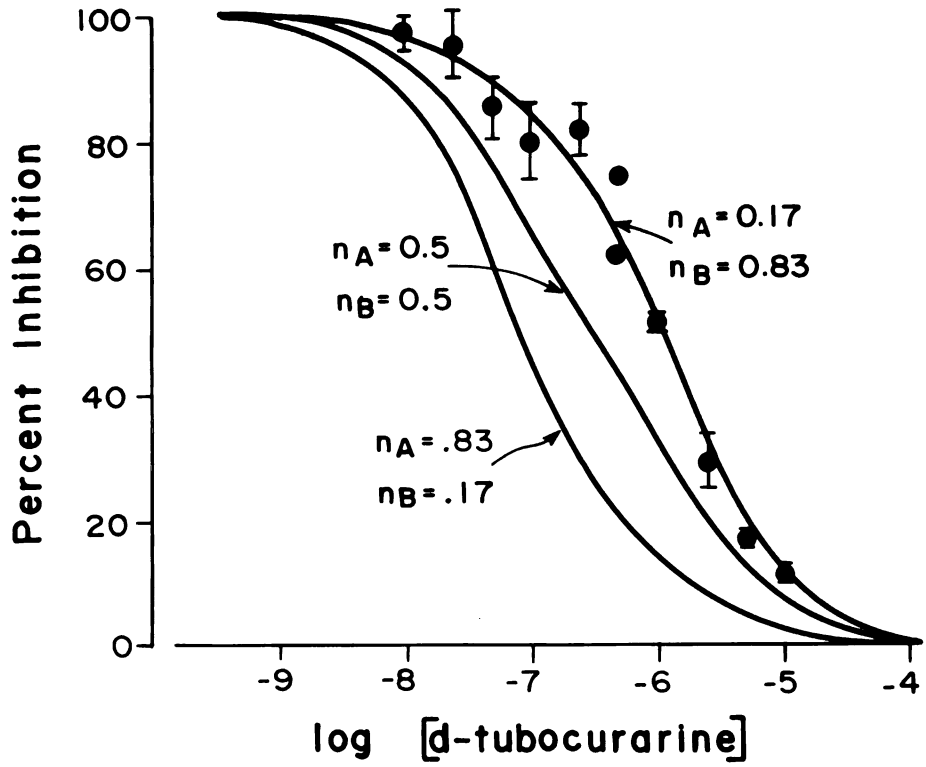

100
80
60
40
20
Percent Association Rate
10-9
10-8
10-7
10-6
10-5
10-4
Log (dTC)

Figure 2-9. Incubation with the myasthenic antibodies specifically blocked toxin binding to the site with high affinity for dTC. Experiments were carried out as described in the legend to Figure 2-7, except that cells were preincubated with 4 mg/ml of the myasthenic antibody for 2 hours at 37 °C. Each *point* represents the mean ± SEM of four determinations. Incubation of cells with the antibody reduced total toxin binding, as assayed under saturating conditions, to 60% of that seen without antibody. The *solid curves* were calculated by using the values for the dissociation constants obtained in Figure 2-7 and by assuming that the 40% sites blocked by the antibody were either all low affinity site (N_A = 0.83, N_B = 0.17), all high affinity sites (N_A = 0.17, N_B = 0.83), or equally shared between the two sites (N_A= 0.5, N_B = 0.5).


100
80
60
40
20
0
Percent Inhibition
n_A = 0.17
n_B = 0.83
n_A = 0.5
n_B = 0.5
n_A = .83
n_B = .17
-9
-8
-7
-6
-5
-4
log [d-tubocurarine]

Chapter 3

The Effects of a Myasthenic Serum on the Acetylcholine Receptors of C2 Myotubes.

II. Functional Inactivation of the Receptor*

* Part of the work presented in this chapter was carried out in collaboration with Drs. A. V. Maricq and S. Hestrin (Department of Physiology, University of California, San Francisco).

Abstract

We have investigated the effect of the antibodies from a myasthenic serum on the physiological properties of acetylcholine receptors (AChRs) in myotubes of a mouse muscle cell line, C2. The antibodies in this serum blocked the binding of ^{125}I-α-bungarotoxin to the myotubes to an extent of about 50%. The antibodies also inhibited the increase in ^{22}Na influx caused by carbamylcholine (CARB). At a concentration of antibody that blocked about 50% of toxin binding, greater than 80% of the AChR-mediated ^{22}Na influx was blocked. The apparent Kd for CARB, estimated from the dose-response curve for ^{22}Na influx, was unaffected. The effect of the antibodies was further examined by patch-clamp recording. In greater than 30% of the patches from antibody-treated cells, no channel activity in response to acetylcholine was seen; in contrast, every patch from control cells showed activity. The channels that were seen after antibody treatment were indistinguishable from those seen in normal cells, both in their single-channel conductance and in the kinetic constants used to describe channel opening and closing. We conclude that the antibodies in this serum inhibit the functional response of AChRs in C2 myotubes to acetylcholine and do so by inactivating individual receptors.

Introduction

Myasthenia gravis is an autoimmune disease in which patients are subject to sporadic weakness and fatigability. The physiological basis of these symptoms is a decreased number of functional acetylcholine receptors (AChRs) in the postsynaptic membrane. Thus, myasthenic endplates show reduced endplate potentials (Elmpuist et al., 1964; Albuquerque et al., 1976a, 1981; Ito et al., 1978; Cull-Candy et al., 1979), a diminished response to iontophoretically applied acetylcholine (ACh) (Albuquerque et al., 1976a; Cull-Candy et al., 1979), and a decreased number of binding sites for α-bungarotoxin (α-BuTx) (Fambrough et al., 1973; Green et al., 1975).

Antibodies to the AChR in the sera of myasthenic patients (Almon et al., 1974) are thought to be responsible for the loss of junctional AChRs. Antibodies are bound to the postsynaptic membrane of the neuromuscular junctions in myasthenic patients (Engel et al., 1977), and injection of antibodies from myasthenic sera into mice induces symptoms of the disease, with reduced miniature endplate potentials (Mepps) and a decreased number of toxin-binding sites (Toyka et al., 1975).

Antibodies to the AChR may exert their effect on the postsynaptic membrane by complement-mediated destruction of the membrane or by increasing the metabolic degradation of AChRs (reviewed by Drachman, 1981; Lindstrom and Engel, 1981). A third

possibility is direct functional block of the AChR. Although many myasthenic sera have antibodies that block the binding of α-BuTx to the AChR (Almon et al., 1974; Bender et al., 1975; Lefvert and Bergstrom, 1977; Mittag et al., 1976; Lefvert et al., 1981; Drachman et al., 1982) and the presence of these antibodies is correlated with the severity of the disease (Drachman et al., 1982), myasthenic sera have rarely been found to inhibit the response of the receptor to ACh (Albuquerque et al., 1976b; Ito et al., 1981; Shibuya et al., 1978; Peper et al., 1981), and in no cases has the inhibition been well characterized.

In the preceding chapter, we have described a serum from a myasthenic patient that is highly specific when assayed against the AChRs from a mouse muscle cell line C2. The antibodies in this serum recognize one of the two α-BuTx binding sites that each receptor possesses. Because this site is presumably close to, or part of, one of the binding sites for ACh, we wished to investigate the effect of these antibodies on the functional response of the C2 receptor to ACh. The antibodies in this serum are of additional interest because they recognize the AChRs at developing but not at adult rat endplates; recent experiments suggest that they bind specifically to AChRs with embryonic-type channels and not to those with adult-type channels (Schuetze et al., 1985). We report here that this antibody blocks the response of AChRs in C2 myotubes to agonists.

Materials and methods

Tissue culture. The C2 mouse muscle cell line was maintained and grown as described previously (Inestrosa et al., 1983, Gu et al., 1985). For experiments with ^{22}Na uptake, myoblasts were plated in 24-well plastic tissue culture plates precoated with collagen, at a density of 3000 cells/well. Forty-eight hours later, the growth medium was replaced with fusion medium. Cells were used for experiments within 4 days after transfer to fusion medium.

For patch-clamp experiments myoblasts were plated on ethanol-cleaned glass coverslips (13 mm in diameter) at a density of 2000 cells per coverslip; after 3 days the medium was changed to fusion medium. Experiments were carried out 3-5 days later.

Measurement of ^{22}Na influx. The medium surrounding the cells was changed to a buffer consisting of 140 mM KCl, 5.4 mM NaCl, 1.8 mM $CaCl_2$, 1.7 mM $MgSO_4$, 1.0 mM Na_2HPO_4, 5.5 mM glucose, 25 mM HEPES, 60 μg/ml bovine serum albumin, pH 7.4 (assay buffer). After equilibration for 20 minutes at room temperature, the cells were further incubated at 4 °C for 30 minutes. ^{22}Na influx measurements were initiated by replacing the buffer with the same medium chilled to 4 °C and containing 5 μCi/ml ^{22}Na and various concentrations of the cholinergic agonist, carbamylcholione (CARB). In a typical experiment, a final concentration 0.1 mM CARB was used to activate the AChRs. After various period of times, uptake measurements were terminated by aspiration of the medium, followed by three quick washes with ice-cold assay buffer containing 0.5 mM d-

tubocurarine to inhibit the activation of channel. Cells in each well were extracted twice with 0.5 ml of 0.1 N NaCl.

The combined extracts were then counted in a Beckman II gamma counter optimized for ^{22}Na. In each experiment, the rate of influx of ^{22}Na in cells pretreated with excess α-BuTx was determined and was subtracted from the rate of influx in cells not treated with α-BuTx; the difference was considered to be the specific influx due to the activation of the receptor.

A first order kinetics analysis was made by plotting the integrated form of the rate equation, $ln\ [Na]^{\infty}_{in}/([Na]^{\infty}_{in}-[Na]^{t}_{in}) = Kt$, where $[Na]^{t}_{in}$ is the internal concentration of ^{22}Na at time t and K is a parameter that is proportional to permeability. If the permeability is constant, the plot yields a straight line with a slope of K.

For the determination of the effects of the myasthenic serum on AChR function, the cells were preincubated with the antibody for 2 hours at 37 °C in a buffer similar to the assay buffer but containing 140 mM NaCl and 5.4 mM KCl. After rinsing the cells once with assay buffer, ^{22}Na influx was measured as described above. The effect of antibody on toxin binding was monitored under the same conditions that were used for the ^{22}Na assay.

Electric recording. Patch-clamp experiments were carried out at room temperature with the cells in a medium containing 135 mM

NaCl, 2 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM KH_2PO_4, 15 mM $NaHCO_3$, 5 mM HEPES, 5 mM Glucose, pH 7.25. The recording pipette was filled with the same solution. In all cases the solutions were filtered through a 0.2 μm filter immediately before use. To examine the effects of antibody on AChR function, the myotubes were incubated for 2 to 3 hours at 37 °C with 3 mg/ml of the antibody in the medium described above. After rinsing to remove excess antibody, the myotubes on coverslips were transferred to the recording chamber and used for 2 to 3 hours.

Pipettes for recording were pulled from acid-cleaned Hematocrit tubing, with an inner diameter of 1.1 mm and an outer diameter of 1.5 mm. The pipettes were coated with Sylgard to within 50 μm of their tips and subsequently microforged to a final tip diameter of 0.5 to 1.0 μm. In the recording solution the DC resistance of the pipette was typically 4 to 8 megohms. The pipette was connected by an Ag-AgCl electrode to the amplifier head stage which was equipped with a 10-gigohm feedback resistor. The bath electrode was 2% agarose in 1 M KCl and connected to ground via an Ag-AgCl electrode. Recording from myotubes were made in the cell-attached patch-clamp configuration with various concentrations of ACh in the pipette. "Outside-out" and "inside-out" patch configurations were used less frequently (Hamill et al., 1981). The current signal was recorded on an FM tape recorder. The frequency response of the recording system was flat up to 4 KHz.

Data analysis. The electrical responses were low-pass filtered at 2 to 3 KHz by an eight-pole Bessel filter and digitized at 10 to 20 KHz. With a double-buffered data acquisition protocol, channel opening events were captured using a trigger value of one-half the unitary current amplitude. Data files consisting of open and closed durations and current amplitudes were organized into histograms with a flexible bin size such that each bin contained at least five events. Exponential or Gaussian functions were fitted to these histograms using the method of nonlinear least squares regression.

The reliability and accuracy of the methods of recording and analysis of the data were verified in the following manner. A computer simulation of a two-state (open-closed) channel was produced by applying command voltages of an exponentially distributed duration to the amplifier with a 1-gigohm resistor across the input. The voltage was adjusted so that the signal-to-noise ratio approximated that found in an experiment. The recording and data analysis applied to the simulated data were the same as those described above (filter set at 3 KHz). With these techniques, time constants greater then 0.06 msec could be estimated without systematic bias.

Antibodies. The antibodies used in this study have been described previously (Hall et al., 1983, 1985). They are derived from a serum taken at plasmaphoresis by Dr. Pater Dau at Children's Hospital in San Francisco, in February 1978. The 40% ammonium sulfate precipitate, after storage for 3 years at Children's Hospital,

was dialyzed in our laboratory against 0.02 M ammonium bicarbonate, lyophilized, and stored at -20 °C. For the ^{22}Na influx experiments, the lyophilized protein was taken up just before use in a buffer similar to the assay buffer but containing 140 mM NaCl and 5.4 mM KCl. For the electrophysiology experiments we used an IgG fraction prepared by DEAE chromatography as described previously (Hall et al., 1985).

Results

CARB-stimulated ^{22}Na influx. In our initial experiments we examined the effects of the myasthenic antibodies on CARB-stimulated ^{22}Na uptake into C2 myotubes as a measure of AChR function. Our assay was modeled on that described by Sine and Taylor (1979) for the non-fusing mouse muscle cell line BC3H-1. Experiments were performed at 4 °C to decrease the rate of receptor desensitization (Catterall, 1975), and the measurement was made in a buffer with a potassium concentration (140 mM) similar to the intracellular potassium concentration to eliminate the secondary effects of voltage changes on ^{22}Na influx. Under these conditions the membrane potential and the potassium equilibrium potential are close to zero, and the large increase in sodium and potassium permeabilities caused by the cholinergic agonist should have little effect on transmembrane voltage. In addition, the voltage-dependent sodium channels are inactivated.

When C2 myotubes were incubated with 100 μM CARB, there was an initial rapid influx of ^{22}Na followed by a slow, steady uptake (Figure 3-1). The initial, rapid phase was completely blocked by α-BuTx and was therefore considered to be due to the AChR. ^{22}Na influx mediated by the AChR was complete within 2 minutes after the addition of CARB. A kinetic analysis (Figure 3-1, *inset*) showed that the CARB-induced permeability was constant for only about 20 sec. The decrease after this time was presumably due to desensitization of the receptor. In all subsequent experiments, incubation were for 10 or 15 sec and the influx was measured for a single time point. Under these conditions, CARB-stimulated ^{22}Na influx was 6- to 10-fold higher in myotubes incubated without α-BuTx than in toxin-treated control cells.

Effects of the myasthenic serum. Incubation of C2 myotubes with the myasthenic serum for 2 hours at 37 °C reduced the ^{22}Na influx induced by CARB. Increasing amount of the antibody gave a progressive inhibition of influx to a maximum of approximately 80% at 8 mg/ml of antibody (Figure 3-2). When inhibition by antibody of toxin binding and ^{22}Na influx was compared in the same experiment, the percentage of inhibition of influx was roughly 2 times the inhibition of toxin binding. Thus, for example, an antibody concentration of 1.25 mg/ml gave 50% inhibition of ^{22}Na influx and 25% inhibition of toxin binding. This is the relation that one would expect if only one of the two toxin-binding sites associated with each receptor was susceptible to the antibody and that blocking one site is enough to inactivate the channel. A plot of the extent of

inhibition of toxin binding versus the extent of inhibition of ^{22}Na influx shows reasonable agreement with the curve predicted by this hypothesis (Figure 3-2B, *inset*).

Effects on the apparent K_d for CARB. Because our assay are done at a constant CARB concentration, one interpretation of our results is that the antibody decreases the affinity of the AChR for CARB. In that case it should be possible to overcome the effect of the antibody by increasing CARB concentration. To test this idea, we compared the concentration curves for CARB in the presence and absence of antibody. Myotubes were incubated in 4 mg/ml of antibody or in buffer for 2 hours at 37 °C, and the ^{22}Na uptake induced by various concentrations of CARB was determined as described above. In the absence of antibody, CARB concentrations of 1 mM or higher were required to achieve the maximal effect, and 50% of the maximum was obtained at a CARB concentration of approximately 0.1 mM (Figure 3-3).

In the presence of 4 mg/ml of antibody, although the maximal ^{22}Na influx was only 20 to 30% of that seen in untreated cells, the concentration curve was similar. Half-maximal influx occurred at about 10^{-4} M and maximal influx was seen at 1 mM. When normalized to the maximal influx as 100%, the curves from experiments with and without antibody pretreatment were superimposable (Figure 3-3). We conclude that the decrease in ^{22}Na influx caused by the antibody is not due to a decrease in the affinity of the AChR for agonist.

Single channel analysis. The decrease in CARB-stimulated ^{22}Na influx caused by the antibody could result from a reduction in the single channel conductance of the AChR, a decrease in the mean channel open time, a reduced frequency of channel opening events due either to fewer functional channels or to a decreased probability of channel opening, or some combinations of the above. To investigate whether these properties were affected by the antibodies we used patch-clamp techniques to carry out a single-channel analysis of the AChR channel following incubation of C2 myotubes with antibody. The properties of the AChR channel in C2 myotubes and a kinetic analysis of their behavior have recently been described (Hestrin et al., 1987).

Channel conductance. Single-channel openings were seen both in control and antibody-treated myotubes. With either 1.0 or 10.0 μM ACh in the recording pipette no obvious effects of the antibodies were seen on channel amplitude or kinetics (Figure 3-4). In most patches, as was true in control myotubes (Hestrin et al., 1987), the histograms of unitary current amplitude reveals only one peak. Occasionally a second minor peak was observed which corresponded to a large current size. These later events were not further analyzed. Plots of mean unitary current versus applied voltage for the major channel showed a linear relationship over a range of 0 to -125 mV (Figure 3-5). The slope conductance for antibody-treated cells was 36.5 +/- 0.8 pS, a value that was not significantly different from the control value of 35.0 +/- 0.5 pS. Thus, under the

ionic conditions used, the antibody does not impair the conductance of the AChR channel.

Channel open time. The channel, once open, conducts normally. Perhaps the decrease in ^{22}Na flux caused by the antibody occurs because the channel stays open for a shorter period of time. To investigate this possibility, we obtained records of channel opening events at a low ACh concentration. Under these conditions, few channel events overlapped, and individual bursts of channel openings were well separated (a burst is defined here as a sequence of channel openings not interrupted by a gap of greater than 0.5 msec (Colquhoun and Hawkes 1981)). The burst duration histograms of channels from both antibody-treated and control cells were fit by two exponentials: a fast component and a dominant slow component (Figure 3-6, *A* and *C*). These two components have been previously interpreted as corresponding to the channel closing rates from the single and doubly liganded open state, respectively (Hestrin et al. 1987). The time constants and the relative proportion of the two components are shown in table I. No significant differences in time constants or relative proportions were found between the two groups except for the fast time constant, which was approximately 2.5-fold longer in the antibody-treated cells.

Channel closed time. The interval between successive channel openings is referred to as the closed time. Analysis of the closed time can yield the binding constants for the agonists and the rate of the conformational change from the closed to the open state. A

decrease in either the binding constants or the isomerization rate would result in a decrease in ^{22}Na influx. The closed time histograms, required three exponentials for a proper fit. This was true for both the control and antibody-treated channels (Figure 3-6, *B* and *D*). As can be seen in Table 3-1, the values for the time constants and the relative power contributions at a low ACh concentration did not significantly differ between the two groups. To test further whether the channel kinetic parameters were affected by the antibody, we examined the closed time histogram of channels exposed to a higher concentration of ACh. As the ACh concentration is raised, the closed time histogram exponential collapse to a single component, the rate of which depends on the ACh concentration and which converges at high ACh concentrations to the rate of isomerization from closed to open channel. At 10 μM ACh, the time constant of the major component was 2.2 msec for channels in antibody-treated cells, a value that was not significantly different from the control value of 2.5 msec (Hestrin et al., 1987). The fact that the multi-exponential functions required to fit the closed time histogram under several different ACh concentrations were not different between antibody and control groups provides an additional assurance, independent of the particular kinetic model chosen, that the underlying kinetics of channel opening had not been changed by the antibody.

Frequency of channel bursts. The kinetic analysis of the AChR channels was performed on records which contained at least 500 channel opening events in several minutes and which did not contain

so many events that channel overlap hindered the analysis. The burst frequencies obtained from antibody-treated channels were therefore not very different from those of controls. The patches selected for kinetic analysis, however, were a minority of the successful patches that we obtained in the case of antibody-treated cells. Although it was difficult to control for differences in the area of the membrane patch or the specific location of the patch on the cell, it was clear, in a series of experiments on antibody-treated cells and sister control cells, that many of the patches from antibody-treated cells showed a reduced number of bursts compared to the control patches (Figure 3-7). In fact, approximately 30% of the patches from antibody-treated cells showed no channel activity over periods of 5 to 10 minutes, a phenomenon that was never observed in patches from control myotubes.

Discussion

Although virtually all myasthenic sera have antibodies directed against the AChR, and many sera have antibodies that block α-BuTx binding to the AChR, few sera have been found that affect the functional response of the receptor molecule to ACh (Nastuck et al., 1959; Namba and Grob, 1969; Albuquerque et al., 1976b, 1981; Harvey et al., 1978; Ito et al., 1978; Peper et al., 1981). There are a few incidental accounts of sera that have inhibitory effects on the response to ACh, but the action of these sera has invariably been found to be weak or partial (Ito et al., 1978; Shibuya et al., 1978; Peper et al., 1981; see also an unpublished experiment reported by

Bevan et al., 1977). The most effective of these was a single example found by Ito et al. (1978) that reduced the amplitude of MEPPs in human muscle by 50% over a period of 5 hours.

The myasthenic antibodies that we have studied have a more dramatic effect. After 2 hours of incubation, they reduced CARB-stimulated ^{22}Na influx in C2 myotubes by at least 80%. Because the antibodies used in our experiments increase the degradation rate of the AChR only slightly and have little effects on the number of AChRs on the cell surface (Gu et al., 1985; see also Chapter 2), the inhibition of ^{22}Na uptake must result from a direct effect on AChR function. Antibodies in this serum also block the toxin binding to the AChR to a maximal extent of about 50% (Figure 3-2; Gu et al., 1985; see also Chapter 2). The inhibition of ^{22}Na uptake occurs over the same range of concentrations of antibodies, and the relation between inhibition of toxin binding and of ^{22}Na uptake suggests that the same antibodies are responsible for both effects (Figure 3-1, *inset*). C2 myotubes have two classes of toxin-binding sites, and the antibodies specifically block one of these (Gu et al., 1985; see also Chapter 2). The fact that almost complete inhibition of ^{22}Na influx can be obtained when only 50% of the toxin sites are blocked is consistent with a model in which both sites are associated with each AChR channel and block of one is sufficient to diminish ion flow through the channel (Sine and Taylor, 1980).

To further characterize the interaction of the antibodies with the AChR, we examined, at the single-channel level, the residual

current that remained after exposure to the antibody. The reduced ^{22}Na influx could arise from altered channel properties resulting in less current being carried per channel, or it could result from a reduction in the number of functional channels.

The single channel traces appeared to be indistinguishable in antibody-treated and control cells. Amplitude histograms revealed only one unitary current peak under either conditions, and the calculated slope conductance for the AChRs in antibody-treated cells did not significantly differ from that of AChRs in control myotubes. Thus, once open, the channel conducts normally.

Although conducting normally, the channel might stay open for a shorter period of time, which would be reflected in the distribution of burst durations, or the channel might stay closed for a longer period of time, which would be seen in the distribution of closed times. Virtually all of the time constants used to describe the kinetics behavior of the AChR channel were unaffected by prior incubation with the myasthenic antibody. Thus, both opening and closing rates, and the agonist binding rates, were unchanged. The only significant difference was a 2.5-fold decrease in the fast component of the burst duration. This decrease would have negligible effects on the macroscopic current measurements and is also in the wrong direction to explain the flux studies. Thus, the decrease in ^{22}Na flux observed as a consequence of incubation with myasthenic serum cannot be due to altered channel function and must result from a decrease in the number of functional channels.

After antibody treatment, we observed an overall decrease in the frequency of channel opening events which is consistent with there being fewer functional channels in the patch (Figure 3-7). Approximately 30% of the patches from antibody-treated cells showed no channel activity, this never occurred with control cells. Because patch areas and conditions are variable, it is possible, for instance, that the presence of antibody changed the physical properties of the membrane, causing it to seal over during recording. Another possible explanation of the difference is that the presence of the antibody caused the AChRs to aggregate, thus denuding most of the membrane of receptors. Immunocytochemical experiments, however, showed a distribution of AChR clusters that appeared similar to that seen in normal cells (Gu et al., 1985; L. Silberstein and Z. W. Hall, unpublished observations). Thus, the simplest explanation is that the antibody decreases ^{22}Na influx by impairing the ability of the channel to open in response to ACh. Recent experiments on the effects of these antibodies on the AChRs in developing rat muscle are also consistent with this interpretation (Schuetze et al., 1985).

The mechanism by which the antibody impairs AChR function is not clear. The antibody blocks one of the two toxin-binding sites and most likely blocks the associated agonist-binding site. If the second binding site is accessible to the agonist, one might predict an increase in the proportion of singly liganded, compared to doubly-liganded, receptor channel openings (Hestrin et al., 1987). However,

no such increase was seen. This suggests that the antibody either prevents agonist binding to receptor, prevents a conformational change required for channel opening, or blocks access of ions to the channel.

The question of the residual activity seen in the presence of the antibody remains to be addressed. An incomplete block has also been seen in experiments on developing rat muscle (Schuetze et al., 1985). One possibility is that not all AChRs have bound antibody. The low titer and limited amount antibody available make it difficult to achieve saturation. A second possibility is that there is a class of channels that is resistant to the antibody. This hypothesis is an attractive one because the antibody used in our experiments do not recognize AChRs at rat endplate with adult-type channel properties (Hall et al., 1985; Schuetze et al., 1985), and AChRs with such properties have recently been reported in cultured cells (Hamill and Sakmann, 1981; Brehm et al., 1982). Recordings from C2 myotubes, however, revealed essentially a single-channel type (Figures 3-4 - 6; Hestrin et al., 1987), the properties of which were unchanged by incubation with the antibody. Thus, if resistant channels are present, they are not distinguishable from susceptible ones by any of the criteria that we have used, including inhibition of toxin binding.

A final possibility is that the antibodies are heterogeneous, and including those that block both toxin binding and function and those that block toxin binding without affecting function. Antibodies in

the latter class are not uncommon and, in some cases, have been reported to be specific for extrajunctional receptors (Albuquerque et al., 1976b).

These observations thus provide striking example of the ability of antibodies in a myasthenic serum to functionally inactivate the AChR. Further experiments will be required to determine the importance of antibodies of this type in the pathophysiology of the disease.

Table 3-1

Channel properties of the AChR in antibody-treated and control C2 myotubes.

	C2 Myotubes	
	Antibody-treated	Control
Conductance (pS)	36.5 ± 0.8[a]	35.1 ± 0.5
Burst duration (msec)	0.34 ± 0.06 (15)[b]	0.13 ± 0.02 (24)
	7.4 ± 0.9 (85)	7.2 ± 1.0 (76)
Closed time duration	0.1 ± 0.03 (15)	0.11 ± 0.01 (15)
(msec)	1.1 ± 0.2 (8)	1.4 ± 0.2 (6)
	120.5 ± 24.7 (77)	81.3 ± 21.8 (79)

[a] Values are the mean ± SEM (N = 7, antibody; N = 6, control). The ACh concentration was either 1.0 or 0.5 μM.

[b] Numbers in parentheses, percentages.

Figure 3-1. Time course of ^{22}Na uptake into C2 cells. The cell cultures were rinsed and transferred to an ice bath for about 30 minutes as described under "Materials and Methods." The buffer was then replaced with the same medium containing 5 μCi/ml of ^{22}Na and 100 μM CARB. The uptake was stopped at various time periods by aspiration and three quick washes. Cells were then extracted twice with 0.5 ml of 0.1 N NaOH and the combined extracts were counted in a gamma counter. In all experiments the uptake in cells pretreated with excess amounts of cold α-BuTx (▲) was subtracted from the total uptake (●) to yield the specific uptake (o). Each point is the average of three experimental determinations. *Inset*, The data are plotted in the integrated form of the first-order rate equation as described under "Materials and Methods" to show that, under the experimental conditions used, the CARB-stimulated ^{22}Na permeability is constant for only about 20 sec.

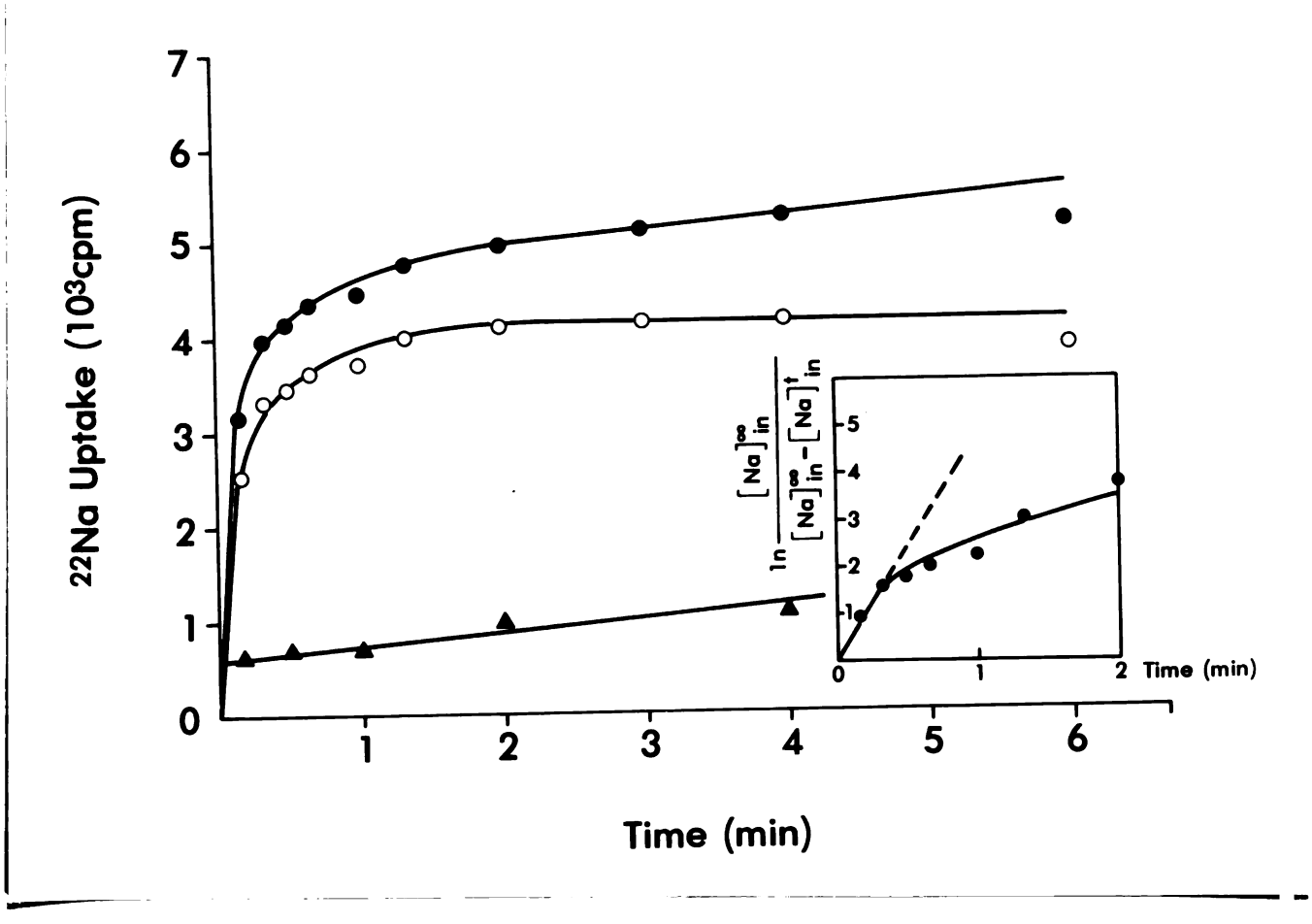

^{22}Na Uptake (10^3cpm)
Time (min)
$\ln \frac{[Na]^{\infty}_{in}}{[Na]^{\infty}_{in} - [Na]^{t}_{in}}$
Time (min)

Figure 3-2. Effects of a myasthenic gravis serum on the binding of α-BuTx to AChRs and on the ^{22}Na influx in C2 myotubes. *A*, The cells were preincubated with different concentrations of the antibody for 2 hours at 37 °C. ^{125}I-labeled α-BuTx was then added to a final concentration of 10 nM and the incubation was continued for another 2 hours. The medium was then aspirated, and the cells were rinsed three times, extracted twice in 0.5 ml of 0.1 N NaOH, and counted in a gamma counter. The binding in the presence of excess amounts of cold α-BuTx was considered to be nonspecific and was subtracted from the total binding. The data were expressed as a percentage of binding in the absence of the antibody. *B*, The cells were preincubated with various concentrations of the antibody for 2 hours at 37 °C, and then the ^{22}Na uptake assay was carried out as described under "Materials and Methods." The uptake in 15 sec was monitored and the percentage of uptake was plotted as a function of antibody concentration. The data are from two independent experiments, and each *point* is the average of two determinations that are indicated by the *error bars*. *Inset*, Percentage of ^{22}Na uptake was plotted as a function of the percentage of α-BuTx-binding sites remaining after antibody treatment. The *straight line* represents the theoretical calculation based on the model in which the binding of the antibody to one of the two binding sites completely blocks receptor function. It is assumed that the ^{22}Na uptake is directly proportional to the number of functioning receptors on the cell surface. The data shown in Figure 2A are reproduced from Chapter 2 and are shown here for comparison

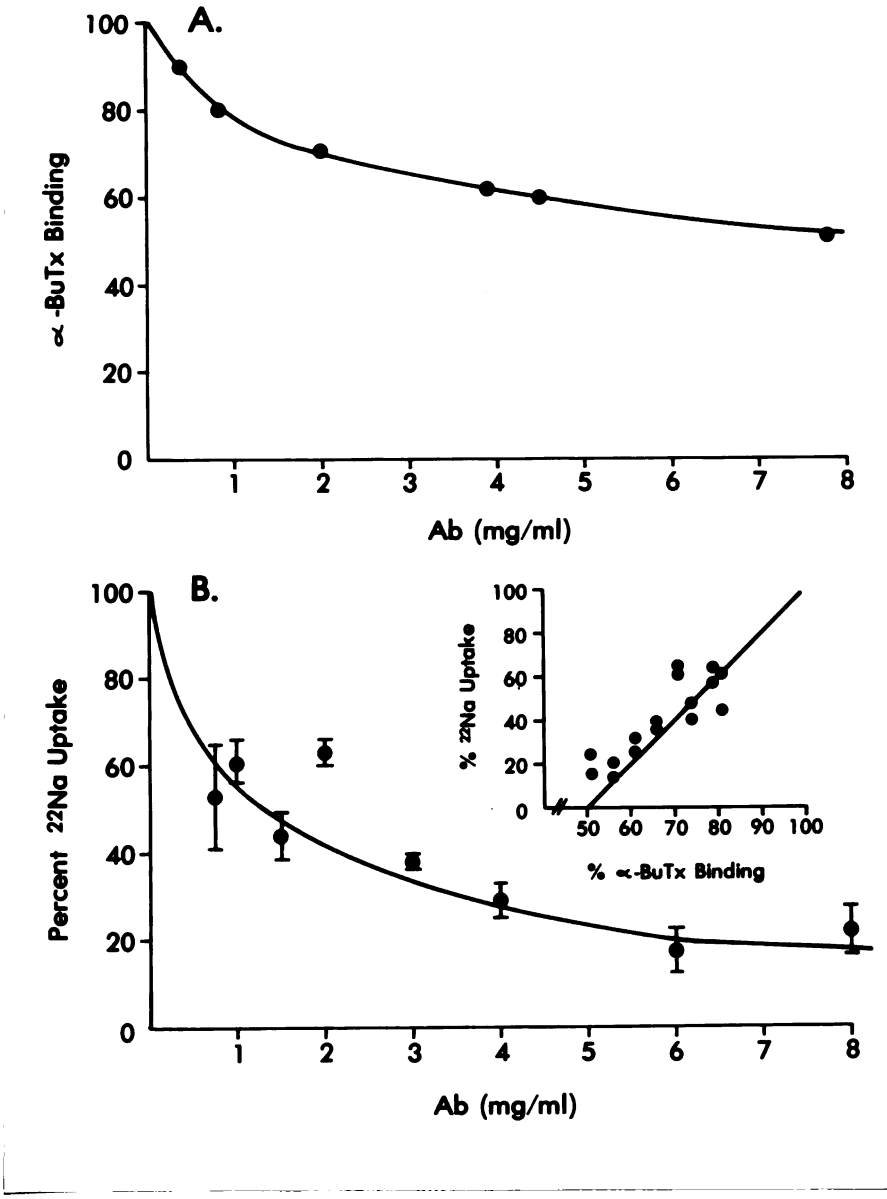

A.
100
80
60
40
20
0
∝-BuTx Binding
1
2
3
4
5
6
7
8
Ab (mg/ml)
B.
100
80
60
40
20
0
Percent 22Na Uptake
1
2
3
4
5
6
7
8
Ab (mg/ml)
100
80
60
40
20
0
% 22Na Uptake
50
60
70
80
90
100
% ∝-BuTx Binding

Figure 3-3. Determination of the apparent K_d of AChR for CARB in normal and antibody-treated C2 cells. Cells were incubated with 4 mg/ml of the antibody (□) or buffer (●, ▲) for 2 hours at 37 °C. The cells were then rinsed and transferred onto ice. ^{22}Na influx was initiated by replacing the medium with the assay buffer containing 5 μCi/ml of ^{22}Na and various concentrations of CARB. After 10 sec, the uptake was stopped and the amount of ^{22}Na influx was determined. Each *point* is the average of two determinations. Different *symbols* represent independent experiments. The data were normalized and were expressed as a percentage of the maximal uptake in each experiment.

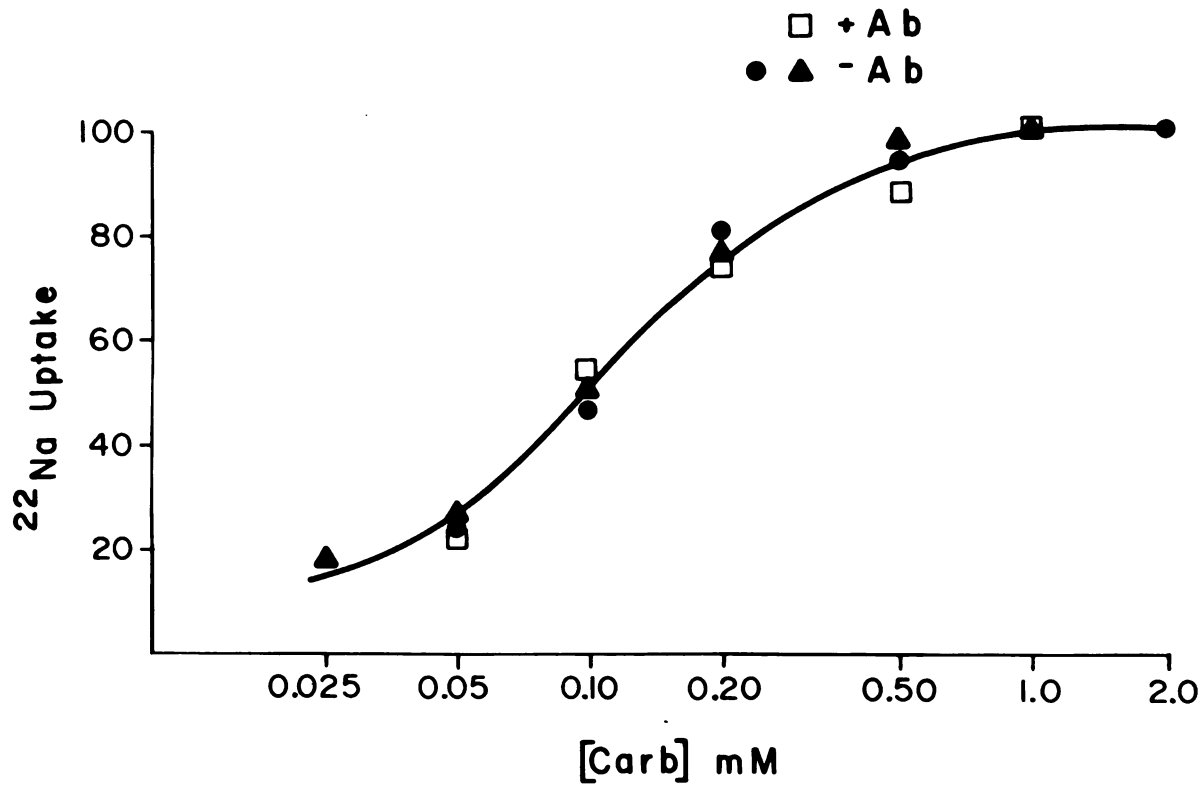

□ + Ab
● ▲ - Ab
100
80
60
40
20
^{22}Na Uptake
0.025
0.05
0.10
0.20
0.50
1.0
2.0
[Carb] mM

Figure 3-4. Single-channel records of ACh-induced current were recorded from patches of C2 myotubes in the cell-attached configuration. The applied membrane potential was -75 mV. The *first* and *third traces* are records from antibody-treated cells, and the *second* and *fourth traces* are from normal cells. The ACh concentration in the pipette was 1 μM in the *first* and *second* traces and 10 μM in the *third* and *fourth* traces.

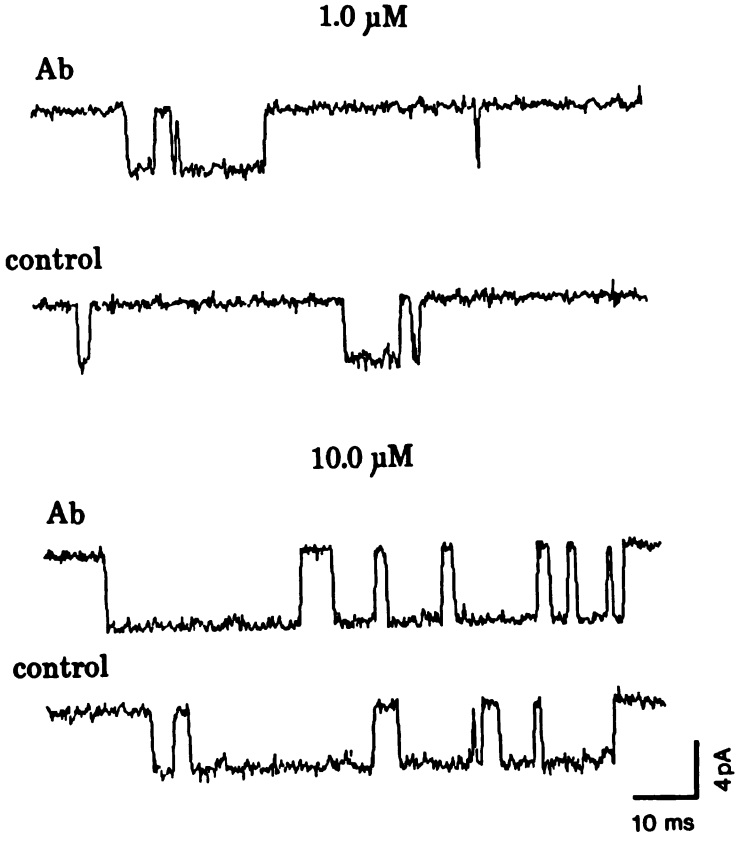

1.0 µM
Ab
control
10.0 µM
Ab
control
4pA
10 ms

Figure 3-5. Current-voltage relationship for the AChR channel. The *lower symbols* are values from an antibody-treated myotube. The slope conductance is 37.2 pS and and the calculated resting potential (assuming a reversal potential of 0 mV) is -65 mV. The *upper symbols* are the control values. The slope conductance is 34.1 pS and the calculated resting potential is -54 mV.

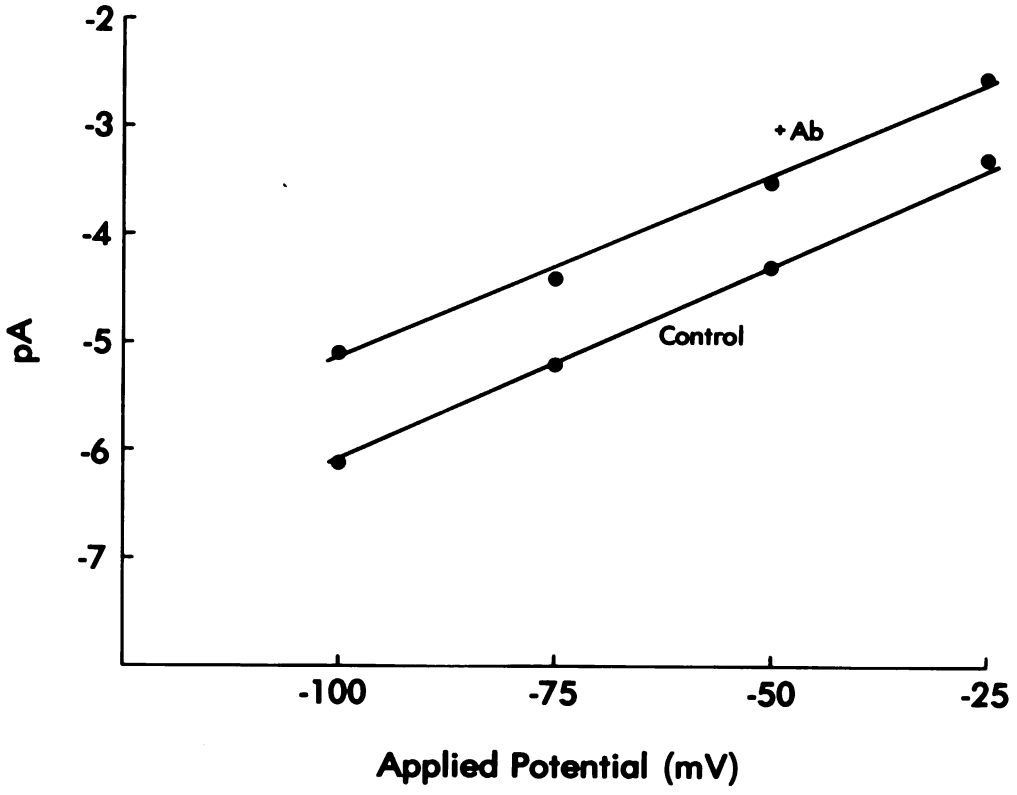

-2
-3
-4
-5
-6
-7
pA
+Ab
Control
-100
-75
-50
-25
Applied Potential (mV)

Figure 3-6. Kinetic analysis of patch clamp current records. The average current size was 4.35 pA (SD, 0.8). *A,* The burst duration histogram was obtained from a patch incubated with 3 mg/ml of a DEAE fraction of the myasthenic antibody and then exposed to 1.0 μM ACh. Openings separated by a gap lasting less than 0.5 msec were considered to belong to the same burst. Two exponentials with the following time constants were required to fit the histogram: 0.20 msec with 24% power and 7.04 msec with 76 % power. *B,* The closed time distribution obtained from the same patch as in *A*. Three exponentials were fit to these data with the following time constants: 0.08 msec with 9.5% power, 0.68 msec with 12.5% power, and 97.08 msec with 78% power. *C,* The burst duration histogram obtained from a control patch. The conditions were as in *A*, except there was no incubation with antibody. Two exponentials with the following time constants were required to fit the histogram: 0.28 msec with 15% power and 10.87 msec with 85% power. *D,* The closed time distribution obtained from the same control patch as in *C*. Three exponentials were fit to these data with the following time constants: 0.15 msec with 7% power, 0.98 msec with 5% power, and 125.0 msec with 88% power.

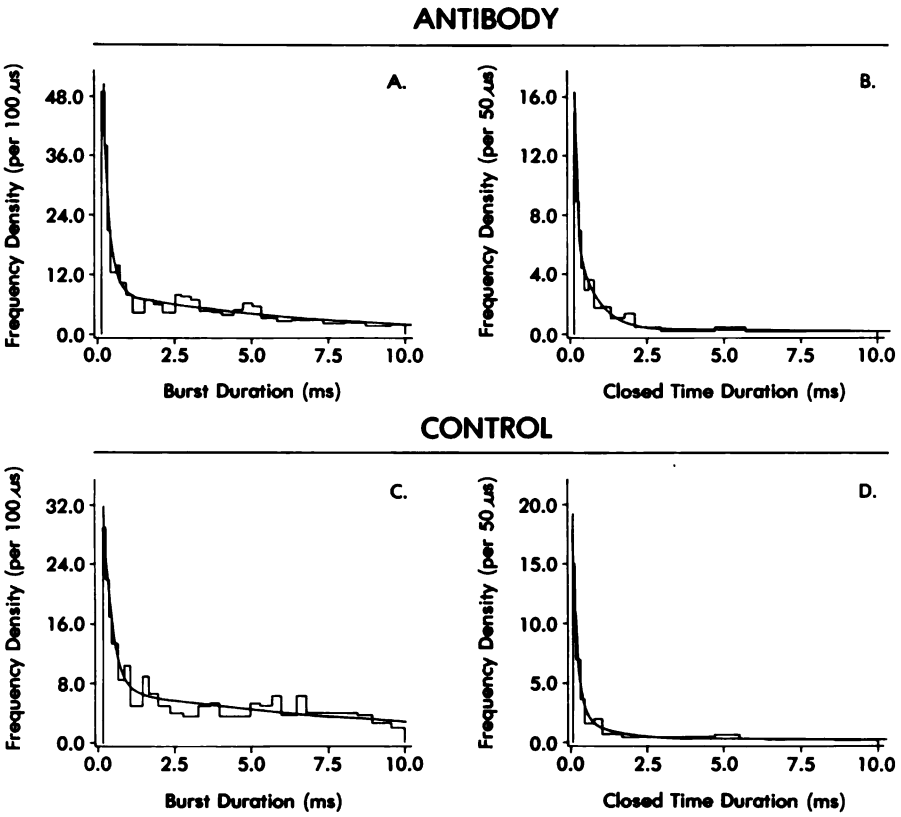
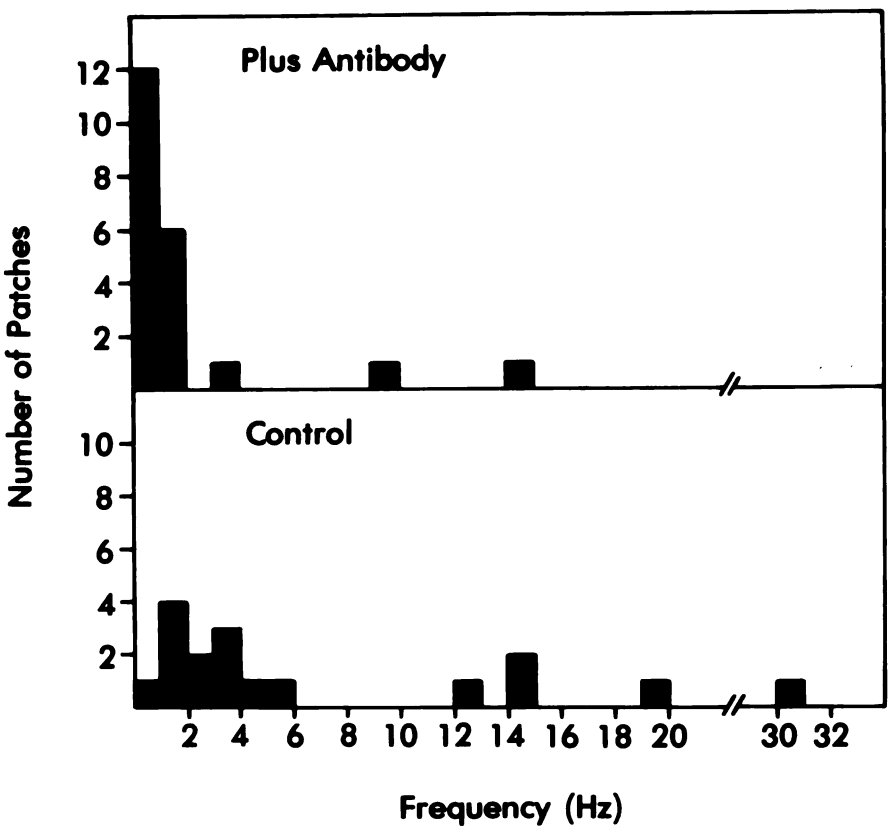

ANTIBODY
A.
B.
Frequency Density (per 100 μs)
Frequency Density (per 50 μs)
48.0
36.0
24.0
12.0
0.0
16.0
12.0
8.0
4.0
0.0
0.0
2.5
5.0
7.5
10.0
Burst Duration (ms)
Closed Time Duration (ms)
CONTROL
C.
D.
32.0
24.0
16.0
8.0
0.0
20.0
15.0
10.0
5.0
0.0

Figure 3-7. A histogram of the frequency of channel burst events in membrane patches from control and antibody-treated C2 myotubes. The frequency data from all patches were put into bins of 1 Hz. The *top panel* shows patches incubated with 3.0 mg/ml of a DEAE fraction of the myasthenic antibody and exposed to 1.0 to 0.5 μM ACh. The *bottom panel* shows patches form control sister cultures. All patches in which no channel activity was observed were included in the 0- to 1-Hz bin.


Plus Antibody
Control
Number of Patches
12
10
8
6
4
2
10
8
6
4
2
2 4 6 8 10 12 14 16 18 20 30 32
Frequency (Hz)

Chapter 4

Immunological Evidence for a Change in Subunits of the Acetylcholine Receptor in Developing and Denervated Rat Muscle

Abstract

We have prepared specific antibodies to γ, δ and ε subunits of the mammalian muscle acetylcholine receptor (AChR), using synthetic peptides with subunit-specific sequences as antigens. AChRs in extracts of developing, adult and denervated rat muscle were equally reactive with anti-δ antibodies, but showed differing reactivities with anti-γ and anti-ε antibodies. The AChRs in normal adult muscle were immunoprecipitated by anti-ε, but not by anti-γ antibodies, whereas AChRs in denervated or embryonic muscles were precipitated by anti-γ antibodies but showed little or no reactivity to anti-ε antibodies. When AChRs at endplates were examined by immunofluorescence, those in neonatal muscle bound only antibodies to γ or δ, but not ε subunit, while those in adult muscles bound only antibodies to ε or δ, but not γ subunit. AChRs at denervated endplates and those at developing endplates between postnatal days 9 and 16 bound all three antibodies. We conclude that the distribution of γ- and ε subunits of the AChR during development and after denervation parallels the distribution of AChRs with embryonic and adult channel properties, respectively.

Introduction

The acetylcholine receptor (AChR) is an oligomeric ion channel protein with the subunit composition of $\alpha_2\beta\gamma\delta$ (McCarthy et al., 1986). In mammalian muscle AChRs are subject to complex regulation of their distribution and function during muscle development and following denervation in the adult (for reviews see Role and Schuetze, 1987; Salpeter, 1987). AChRs are diffusely distributed on the muscle fiber surface early in development, but upon innervation become concentrated at the site of motor nerve contact (Bevan and Steinbach, 1977). After innervation, the density of AChRs in the extrajunctional membrane decreases and in the rat they become virtually undetectable during the first week after birth (Bevan and Steinbach, 1977). When adult muscle is denervated, new AChRs appear in the muscle membrane outside the endplate (Miledi, 1960; Brockes and Hall, 1975).

The characteristics of AChR channels also change during development. AChRs of neonatal muscle have a smaller single channel conductance and a longer apparent mean channel open time than do AChRs at adult endplates. The adult form appears during the first postnatal week in the rat and replaces the neonatal form within about two weeks. During the time of transition, endplates have both forms of channels (Sakmann and Brenner, 1978; Fischbach and Schuetze, 1980; Vicini and Schuetze, 1985).

Immunological experiments suggest that the difference in channel properties between AChRs at neonatal and adult endplates is the result of a structural change in the receptor itself. Antibodies from the serum of a patient with myasthenia gravis distinguish AChRs with the two types of channel properties *in situ* (Schuetze et al., 1985), and after receptor solubilization and purification (Hall et al., 1985). Monoclonal antibodies that distinguish between AChRs at adult endplates and those in the extrajunctional membrane of denervated muscle have also been isolated (Whiting et al., 1986; Souroujon et al., 1985).

Numa and colleagues have recently provided important evidence suggesting that the transition in channel properties during development results from a change in the subunit structure of the AChR in which γ subunit is replaced by a related subunit, ε (Takai et al., 1985). They have shown by Northern blotting that mRNAs for γ and ε subunits are differentially expressed during development of calf and rat muscle (Mishina et al., 1986; Witzemann, 1987), with γ expressed at early stages and ε at later stages. Moreover, they demonstrated that oocytes injected with mRNAs derived from cDNA sequences for bovine α, β, γ, and δ subunits expressed nicotinic AChRs with channel properties similar to those of bovine embryonic AChRs, while oocytes injected with mRNAs for α, β, ε, and δ subunits expressed AChRs with channel properties similar to those of adult AChRs (Mishina et al., 1986). These results strongly suggest that a change in the subunit composition of the AChR occurs during

development and is responsible for the transition in channel properties on AChRs at developing endplates.

To examine directly the subunit composition of AChRs, we used synthetic peptides to prepare specific antibodies for γ, δ, and ε subunits. We then used these antibodies to characterize AChRs in extracts and at the endplates of developing and denervated rat muscle. We report: 1) that γ-specific antibodies recognize only AChRs derived from embryonic and denervated muscle, whereas ε-specific antibodies recognize those from normal adult muscle; 2) that AChRs at developing endplates switch from anti-γ immunoreactivity to anti-ε immunoreactivity during the first and second postnatal weeks; 3) that endplates in denervated muscle retain their immunoreactivity to ε-specific antibodies, but also become immunoreactive to anti-γ antibodies within a few days after nerve section.

Materials and Methods

Peptides. Peptides corresponding to sequences from AChR subunits were chemically synthesized by the UCSF Biomolecular Resource Center. Seven sequences were chosen based on published cDNA sequences for three subunits, γ, δ, and ε. The γ and δ cDNA sequences are from a mouse muscle cell line (Lapolla et al., 1984; Yu et al., 1986), and the ε sequence is from calf muscle (Takai et al., 1985). Sequences were chosen that are subunit-specific, that are outside the postulated membrane-spanning regions, and thus more

likely to be accessible to antibodies, and that are not interrupted by known introns in the genomic sequence. The individual AChR sequences used for each peptide is given in Table 4-I. A cysteine residue was added to the N terminal of each peptide to aid in coupling. The purity of the peptide was determined by HPLC and ranged from 63% to 85%. The content of free cysteine sulfhydryl group of each peptide, estimated by Ellman's method (Sedlak and Lindsay, 1968), ranged from 32% to 70%. The peptides were used without further purification.

For immunization, the peptides were coupled to a carrier, soybean trypsin inhibitor (STI; Sigma Chemical Co., St. Louis, MO), through peptide cysteine residues, using sulfo-m-maleimido-benzoyl-N-hydroxysuccinimide ester (sulfo-MBS; Pierce Chemical Co., Rockford, IL) as the coupling reagent. Sixty milligrams of STI in 3 ml of 50 mM KH_2PO_4 (pH 7.0) was mixed with 1.5 ml of 20 mg/ml sulfo-MBS in the same buffer and stirred on ice for 30 minutes. The modified STI was separated from unreacted sulfo-MBS by gel filtration on a P-2 column equilibrated with the same phosphate buffer. A 10 mg aliquot of the STI-sulfo-MBS was then reacted with 10 mg peptide in 5 ml of 50 mM K_2HPO_4 (pH 7.0) and stirred for 30 minutes at room temperature. Coupling efficiency was estimated by the disappearance of free sulfhydryl groups and ranged from 60% to 100%. Rabbits were immunized with the unpurified reaction mixture as described below.

Antibody preparation. Rabbits were initially immunized with 1 mg peptide-coupled STI in complete Freund adjuvant (1:1) given by intramuscular and subcutaneous injections. Boosting injections of 1 mg of conjugated peptide in incomplete Freund adjuvant (1:1) given by the same routes were then made 2 weeks after the first injection and at 3 week intervals thereafter. Animals were bled 2 weeks after each boosting injection.

Unless otherwise stated, all antibodies used in these experiments were affinity-purified from the antisera using peptides coupled to Sepharose 4B as affinity column. Bound antibodies were eluted with 0.1 M glycine-HCl (pH 2.5), neutralized immediately with concentrated Tris buffer, and dialyzed against several changes of PBS. The reactivities of the antisera against peptides were determined in a two-step ELISA using goat anti-rabbit IgG coupled to horseradish peroxidase (Cappel, Westchester, PA) (Tamura et al., 1983).

Immunoprecipitation of Solubilized AChRs. AChRs from normal and denervated adult or embryonic (ED 16-19) rat muscle were solubilized and partially purified as previously described (Hall et al., 1985), using cobratoxin-Sepharose affinity chromatography as the single purification step. Denervated lower leg muscle was obtained from rats whose sciatic nerve had been cut 2 weeks previously. Partially purified AChR was labeled with ^{125}I-α-BuTx in 10 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100 (pH 7.4), and reacted with antibody for 2 hours at room temperature.

Fifty microliters of a 20% suspension of immobilized protein A (Pansorbin; Calbiochem, La Jolla, CA) in 150 mM NaCl, 5 mM EDTA, 50 mM Tris, 1% Triton X-100, 0.02% NaN_3 (pH 7.4), was added, and the incubation continued for 30 minutes. After centrifugation, the pellets were washed with the same buffer and counted in a γ counter. The total amount of AChR used in each experiment was determined using a DEAE filtration assay (Brockes and Hall, 1975) and was expressed in terms of α-BuTx binding sites.

Immunocytochemistry. Immunofluorescent staining of muscle endplates was carried out on 4-6 μm thick cryostat sections of rat diaphragm muscles according to the general procedures described previously (Sanes and Hall, 1979; Hall et al., 1985). Sections were incubated with primary antibody solution for 2 hours at room temperature and, subsequently, with a mixture of fluorescein-conjugated goat anti-rabbit antibody (Cappel Laboratories, Westchester, PA) and rhodamine-conjugated α-BuTx. In some experiments, the sections were pretreated with 50 mM ethylamine-HCl (pH 11.0) for 3-5 minutes before staining with antibody.

In Vitro Translation and Immunoprecipitation. *In vitro* translation of AChR subunits from SP6 RNA polymerase-generated transcripts was performed using the rabbit reticulocyte lysate system (Pelham and Jackson, 1976). Capped transcripts were synthesized using the SP6 RNA polymerase system described by Krieg and Melton (1984), using as templates plasmids containing the cloned cDNA encoding α, β, γ, and δ subunits of the mouse muscle

AChR obtained from the BC3H-1 cell line (generously given to us by L. Yu and N. Davidson, California Institute of Technology). Each subunit (α, β, γ, and δ) was translated separately in the presence of tran ^{35}S label (ICN Radiochemicals, Irvine, CA). The translation product was then mixed and solubilized in a buffer containing 1% Triton X-100, 10 mM N-ethylmaleimide, and 1 mM sodium tetrathionate. An identical mixture was then precipitated with 1 μl of each of the antibodies to be tested, as described for solubilized AChR. The precipitate was solubilized in SDS-PAGE sample buffer (Laemmli, 1970) and subjected to electrophoresis and fluorography.

Results

To obtain subunit-specific antibodies, we made antisera to synthetic peptides derived from the deduced amino acid sequences of γ, δ and ε subunits of the mammalian muscle AChR (Table 4-1). The amino acid sequences for γ and δ subunits are from the mouse muscle cell line BC3H-1; the ε sequence is from calf muscle. Peptides were chosen from regions that are unique for each subunit. Three were from the C-termini of γ, δ and ε subunits, respectively; two, from γ and ε subunits, were from the long cytoplasmic loop between hydrophobic regions M3 and the amphipathic region; and two, also from γ and ε subunits, were from a region just proximal to hydrophobic region M1 (Numa et al., 1983; Finer-Moore and Stroud, 1984).

Each antiserum was initially tested in an ELISA for reactivity against the immunizing peptide and for specificity. Six of the seven peptides elicited antisera that were active against the original peptide (Table 4-1). None of these showed activity in the ELISA against any peptide other than the one used to generate the serum. Thus each serum is specific for a peptide that corresponds to a distinct sequence among the three subunits.

Immunoprecipitation of AChR subunits translated *in vitro*. To test the subunit specificity of these antibodies, we have used them to precipitate the products of *in vitro* translations for α, β, γ and δ subunits. mRNA for each of the four subunits generated from the corresponding mouse cDNAs in SP6 vectors were translated individually using the reticulocyte lysate system as described in Materials and Methods. The translation products were then solubilized and mixed. Aliquots of this mixture were precipitated with anti-γ, anti-δ, or anti-ε antibodies. As shown in Figure 4-1, anti-γ 485 antibodies precipitated only γ subunit polypeptides and anti-δ 486 antibodies precipitated only δ subunit products; Because the cDNA for ε subunit was not available, we were unable to test for antibody binding to ε products, but anti-ε 360 antibodies did not precipitate α, β, γ, or δ translation products to any significant extent (Figure 4-1, lane 7). Thus, insofar as it could be tested, each of the anti-peptide antibodies was specific for the subunit from which the peptide was derived.

Activity against solubilized AChR. Affinity-purified antibodies from each of the six sera were then tested for their activity against the native AChR by immunoprecipitation of toxin-receptor complex. Partially purified AChRs from either normal or denervated adult rat leg muscles were labeled with ^{125}I-α-BuTx and precipitated with the antisera. To compare the titers of the antibodies against the AChR from normal and denervated muscles, comparable amounts of the receptor from the two preparations were used in each case.

Of the six active sera, three recognized receptors purified from normal or denervated rat muscle, or both. As shown in Figure 4-2, anti-δ 486 antibodies immunoprecipitated AChRs both from normal and from denervated adult muscles; these antibodies were approximately equally active against receptors from both tissues. Anti-γ 485 antibodies precipitated AChRs from denervated muscle, but did not recognize AChRs from normal adult muscle. Anti-ε 360 antibodies, in contrast, precipitated normal muscle AChRs but were only weakly active against AChRs from denervated muscle. None of the other antibodies precipitated receptor from either of the two AChR preparations. The immunoprecipitations were incomplete in all cases, presumably due to the low concentration of receptor, and possibly also because of partial degradation of receptor subunits. Nevertheless, the differences in the initial slopes allow an estimate of the relative subunit concentration (Reiness and Hall, 1981).

These results demonstrate first that several of the antibodies to subunit-specific sequences bind to the intact AChR, and second

that the antibodies to ε and γ peptides distinguish the AChRs of denervated muscle from those of normal adult muscle. These antibodies also distinguish the AChRs of embryonic muscle from those of normal adult muscle. When anti-γ and anti-ε antibodies were incubated with partially purified AChRs that were from embryonic (ED 16-19) rat muscle, receptor was precipitated with the anti-γ 485 antibodies, but virtually no reactivity of the AChR with the anti-ε 360 antibodies was seen (Figure 4- 3).

AChRs in normal adult muscle are located almost entirely at the endplate, while most of those in denervated and embryonic muscle are in the extrajunctional membrane (Berg et al., 1972). Our experimental results thus suggest that AChRs at the endplates of normal adult muscles contain ε subunit, while those in the extrajunctional membranes of denervated and embryonic muscles contain little or none; conversely, the γ subunit appears to be a component of extrajunctional AChRs, but not of those at normal adult endplates. Because the endplate AChRs in denervated and embryonic muscles form such a small fraction of the total receptor, it is difficult to infer their properties from experiments such as these. One explanation, however, for the small amount of AChR precipitated from the denervated preparation by anti-ε antibodies (Figure 4-2c), is that AChRs containing ε remain at the denervated endplate (see below). To test this hypothesis, denervated rat diaphragm muscle was dissected into endplate-rich and endplate-free segments, and AChRs in extracts of each were tested with anti-ε antibodies. The results in the two cases were indistinguishable: a

small amount of AChR was precipitated from both extracts (Figure 4-4). The reactivity of denervated muscle AChR with anti-ε antibodies thus cannot be attributed solely to endplate AChRs.

Endplate AChRs in developing and denervated muscle. To determine the properties of AChRs at endplates in developing and denervated muscles, we used these antibodies in immunocytochemical experiments. Cryostat sections of endplate-rich regions of diaphragms taken from 4-, 9-, or 16-day postnatal rats and from normal and denervated adult rats were incubated with anti-ε, anti-γ or anti-δ antibodies. The sections were then stained with a mixture of rhodamine-conjugated α-BuTx to identify endplate AChR clusters and FITC-conjugated second antibody.

In developing muscle a striking change in antibody binding was observed in the first and second postnatal weeks. As shown in Figure 4-5, anti-γ 485 antibodies strongly stained endplates from 4-day old animals, weakly stained endplates from 9-day and 16-day diaphragms, and did not detectably stain adult endplates. In contrast, anti-ε 360 antibodies did not stain endplates from 4-day animals, but strongly stained 9-day, 16-day and adult endplates. A similar pattern of staining was also observed with an antibody to another ε sequence, anti-ε 459, which was inactive in immunoprecipitating solubilized AChR (data not shown, see Table 4-I). The staining of endplates by anti-γ 485 antibodies required brief treatment of the sections with an alkaline solution (pH 11.0), which has been shown to alter the interaction of the AChR with other

proteins (Bloch and Froehner, 1987). Such treatment did not change the pattern of the staining by anti-ε antibodies but did seem to increase the intensity of the staining (Y. Gu and Z. W. Hall, unpublished observations). Staining of endplates by antibodies to peptides from all three subunits was blocked by the appropriate peptides, and was thus specific (data not shown). These experiments demonstrated for the first time that the AChR at endplates changes during development from a form that is reactive with anti-γ antibodies to one that is reactive with anti-ε antibodies.

Anti-δ 486 antibodies reacted with endplates at all ages. The accessibility of the AChR to these antibodies, however, seemed to change during development. AChRs at day 9 and older endplates reacted readily with the antibody, but detection of antibody binding in day 4 endplates required brief pretreatment of the sections with an alkaline solution (pH 11.0) (Figure 4-6).

When endplates from denervated muscles were examined, a change in immunoreactivity was also seen (Figure 4-7). Within 5 days after denervation, immunoreactivity for γ subunit was detected at endplates. During the same period, anti-ε immunoreactivity remained at the endplates; thereafter endplates exhibited both anti-γ and anti-ε immunoreactivities for at least 40 days after nerve section, the longest time examined. AChRs at denervated endplates at all stages were stained by anti-δ antibodies without pretreatment.

Discussion

We have used antibodies specific for γ, δ and ε subunits of the mammalian muscle AChR to demonstrate a change in the subunit composition of AChRs at endplates during development and after denervation. Using muscle extracts, we have also shown that the subunit composition of the AChRs in the extrajunctional membrane of embryonic and denervated muscles differs from that found at mature endplates.

The validity of our experiments depends upon the specificities of the antibodies that we have used. To obtain these antibodies we used as antigens synthetic peptides whose sequences were derived from regions of the deduced amino acid sequence that were unique for each subunit. Antibodies were purified from the resulting sera by affinity chromatography and their specificities demonstrated in several ways. First, antibodies from each serum were specific for their respective immunizing peptides in ELISA. Second, anti-δ and anti-γ antibodies immunoprecipitated only their corresponding subunits from a mixture of α, β, γ and δ polypeptide chains produced by translation *in vitro*. Neither γ nor δ translation products was precipitated by anti-ε antibodies. In other experiments, we have also shown that γ and δ antibodies recognize distinct bands in Western blots of AChRs obtained from embryonic, neonatal and denervated adult rat muscle and C2 myotubes (see Chapter 5; Y. Gu & Z. W. Hall, unpublished experiments). Third, each of the antibodies bound to AChRs in soluble extracts of muscle. The relative

activities of the three antibody preparations, however, were different when measured against AChRs extracted from denervated or embryonic muscles as compared to AChRs extracted from adult muscles. Thus each antibody preparation must recognize different determinants in the intact receptor. Finally, the observation that neonatal, but not adult endplates were stained by antibodies to γ subunit, and that adult, but not neonatal endplates were stained by antibodies to ε subunit, suggests that there is essentially no cross-reactivity between these two antibody preparations.

Immunoprecipitation and immunocytochemical experiments with these antibodies suggest that AChRs in developing muscles form two types, those containing γ subunit and those containing ε subunit. AChRs in the extrajunctional membranes and at the endplates of neonatal muscles contain the γ, but not the ε subunit, while AChRs at the endplates of adult muscles contain the ε subunit, but not the γ subunit. It has been shown in bovine and rat muscles that γ mRNA is expressed only at early stages of muscle development, whereas ε mRNA is expressed only at later stages (Mishina et al., 1986; Witzemann et al., 1987). Thus, there is a transition during muscle development from AChRs containing γ subunit (γ-AChR) to those containing ε subunit (ε-AChR).

Our results are also consistent with the oocyte injection experiment of Mishina et al. (1986) showing that AChRs containing γ and ε subunits, respectively, constitute two classes of AChRs differing in their channel properties. In both developing and

denervated muscle, there is a good correspondence between the presence of γ- or ε-AChRs observed in our experiments and the presence of AChRs with embryonic or adult channel properties, respectively, found in electrophysiological experiments (Sakmann and Brenner, 1978; Fischbach and Schuetze, 1980; Vicini and Schuetze, 1985; Henderson et al., 1987). The one possible exception is in adult muscles, in which small amounts of AChRs with embryonic channel properties have recently been reported (Brehm and Kullberg, 1987), but at levels (< 3%) that are too low to be detected in our experiments.

In denervated muscle, our results clearly show that the major receptor species contains γ subunit. Further, anti-ε immunoreactivity is present at the same level in regions of muscle without endplates as in endplate-rich regions. Although the level of reactivity in these experiments is low, our results are consistent with recent electrophysiological studies showing that AChRs with adult channel properties are equally distributed throughout the membrane of denervated muscle fibers (Henderson et al., 1987; Witzemann et al., 1987). A comparison of the initial slopes of the immunoprecipitation curves (Reiness and Hall, 1981) in Figure 4-3 suggests that ε-AChR, if present, is less than about 15% of the total, consistent with electrophysiological measurements (Henderson et al., 1987; Witzemann et al., 1987).

The presence of ε subunit in denervated muscle is seen much more clearly by immunofluorescence at endplates. When denervated

endplates are examined by immunofluorescence, both anti-γ and anti-ε immunoreactivities are seen for at least 40 days. Their relative proportion, however, is difficult to estimate from immunofluorescence. A population of AChRs with adult channel properties, presumably corresponding to ε-AChR, has previously been observed to persist at endplates after denervation (Henderson et al., 1987; Sellin and Thesleff, 1981; Brenner and Sakmann, 1983).

Since the turnover rate of preexisting AChRs at mouse and rat endplates increases after denervation (Levitt and Salpeter, 1981; Salpeter et al., 1986; Bevan and Steinbach, 1983), no more than 0.1% of the AChRs that were present at the time of denervation should remain after 40 days. Thus the ε subunit that we see must have been made after denervation. Once initiated, the synthesis of ε subunit continues in spite of muscle inactivity and removal of the motor nerve terminal. The appearance of γ subunit at denervated endplates along with ε suggests that the endplate itself does not determine the specificity of the AChR that is localized there.

In contrast to γ and ε subunits, the δ subunit appears to be present throughout development, as anti-δ antibodies stain endplates at all ages. This finding is of interest since the Northern blot results of Mishina et al. (1986) raise the possibility that a transcriptional change in δ might also occur during development. If such a change occurs, the sequence recognized by anti-δ 486 antibodies does not appear to be affected. Curiously, the accessibility of the δ subunit to these antibodies does appear to be

altered. AChRs at endplates in neonatal muscle are not well-stained until after extraction at alkaline pH, a treatment that alters the interaction of peripheral membrane proteins with the AChR (Bloch and Froehner, 1987). The altered accessibility of the C-terminal of the δ chain could be related to the change in its neighboring subunit; it could also be related to the appearance of folds in the subsynaptic membrane (Teravainen, 1968).

We have previously reported that antibodies in a myasthenic serum recognize AChRs with the channel properties of γ-AChR, but not those of ε-AChR (Schuetze et al., 1985; Hall et al., 1985). Although the basis of the distinction that these antibodies make is unknown, they have similar specificity to the anti-γ antibodies described here. Thus they could be specific for γ subunit or for an epitope on a neighboring subunit that is influenced by γ. Antibodies that bind specifically to AChRs in denervated or embryonic rat muscle, but not to those at adult rat endplates, are common in myasthenic sera (Almon and Appel, 1975; Weinberg and Hall., 1979; Dwyer et al., 1981). The difference in subunit structure between embryonic and adult AChR forms in the rat could explain these immunological differences. Immunological differences between AChRs from normal and denervated human muscles have also been detected (Whiting et al., 1986), suggesting that they may also differ in subunit structure.

Table 4-I. Amino Acid Sequences of the Synthetic Peptides and the Properties of Anti-peptide Antibodies.

			Activity against		
		Antibody		Solubilized	AChR
Peptide	Sequence[a]	Designation	Peptide	AChR	*in Situ*
γ 197-207	DSVAPAEEAGHQ	-	-	-	-
γ 345-354	VQDARFRLQN	-	+	-	-
γ 485-497	FPGDPRPYLPLPD	Anti-γ 485	+	+	+
δ 486-496	FSYSEQDKRFI	Anti-δ 486	+	+	+
ε 193-210	VIRRHDGDWAGGP GETD	-	+	-	-
ε 360-369	RAEELILKKP	Anti-ε 360	+	+	+
ε 459-471	FNRVPQLPYPPCM	Anti-ε 459	+	-	±

[a] The deduced amino acid sequences are from Lapolla et al., (1984) (δ), Yu et al., (1986) (γ), and Takai et al., (1985) (ε).

Figure 4-1. Subunit specificity of the antibodies. mRNAs for mouse α, β, γ, and δ subunits were translated individually in an *in vitro* translation system as described. An aliquot from each reaction mixture was taken and subjected to SDS-PAGE (Lanes 1-4, for α, β, γ, and δ, respectively). The rest was solubilized, mixed, and immunoprecipitated with anti-γ 485 (lane 5), anti-δ 486 (lane 6), or anti-ε 360 (lane 7) antibodies and the precipitate analyzed on SDS-gels as described. The figure shows an autoradiograph of the gel. Numbers on the left correspond to molecular mass in kilodaltons.

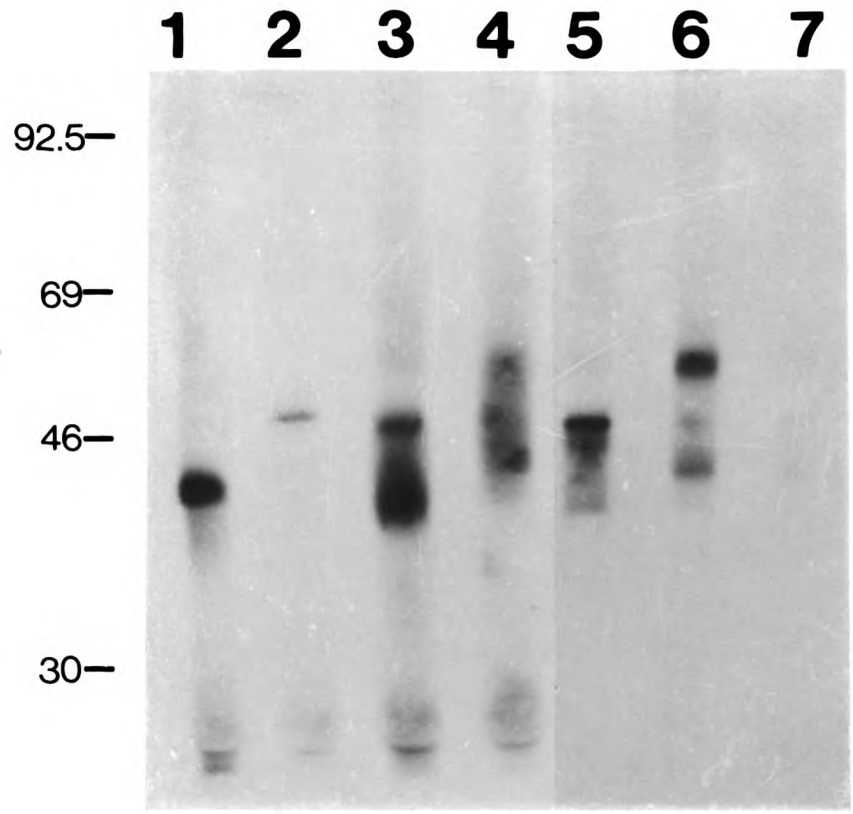

1
2
3
4
5
6
7
92.5
69
46
30

Figure 4-2. Specificity of the anti-peptide antibodies for AChRs from normal and denervated rat muscle. AChRs partially purified from normal (□) or denervated (■) adult rat leg muscle were labeled with ^{125}I-α-BuTx and then incubated with the indicated amount of anti-δ 486 (A), anti-γ 485 (B), or anti-ε 360 (C) antibodies in a total volume of 25 μl, followed by 50 μl of Pansorbin. The amount of receptor immunoprecipitated was expressed as a percentage of total receptor in the reaction mixture. Nonspecific precipitation in the absence of the first antibody was subtracted from each point. For each antibody, the same amount of the two receptor preparations was used. Values of 100 % in (A), (B), and (C) represent 207, 70, 63 fmol of AChR, respectively.

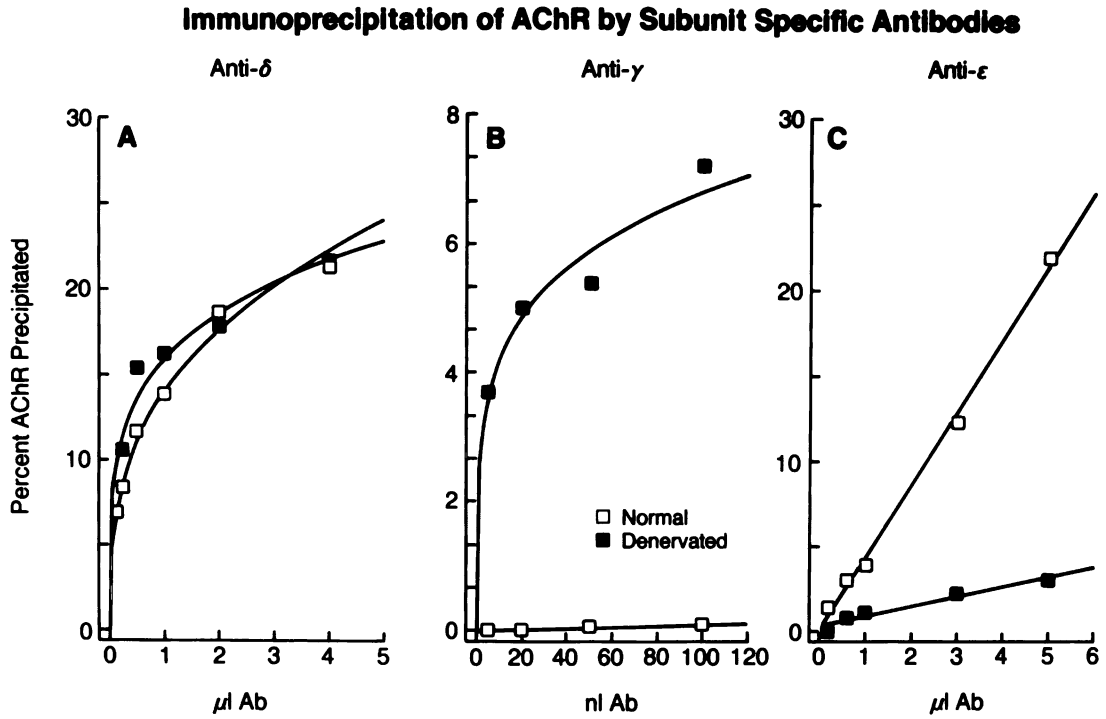

Immunoprecipitation of AChR by Subunit Specific Antibodies
Anti-δ
Anti-γ
Anti-ε
A
B
C
Percent AChR Precipitated
μl Ab
nl Ab
μl Ab
Normal
Denervated

Figure 4-3. Immunoprecipitation of embryonic rat muscle AChR by anti-γ 485 and anti-ε 360 antibodies. AChRs purified from 16-19 day embryonic rat muscle were labeled with ^{125}I-α-BuTx and immunoprecipitated with the indicated amount of anti-γ 485 (A) or anti-ε 360 (B) antibodies in a total volume of 25 μl, followed by 50 μl of Pansorbin. The amount of receptor immunoprecipitated was expressed as a percentage of total receptor in the reaction mix, which was 86 fmol. Nonspecific precipitation in the absence of primary antibody was subtracted from each point.

Immunoprecipitation of Embryonic Muscle AChR

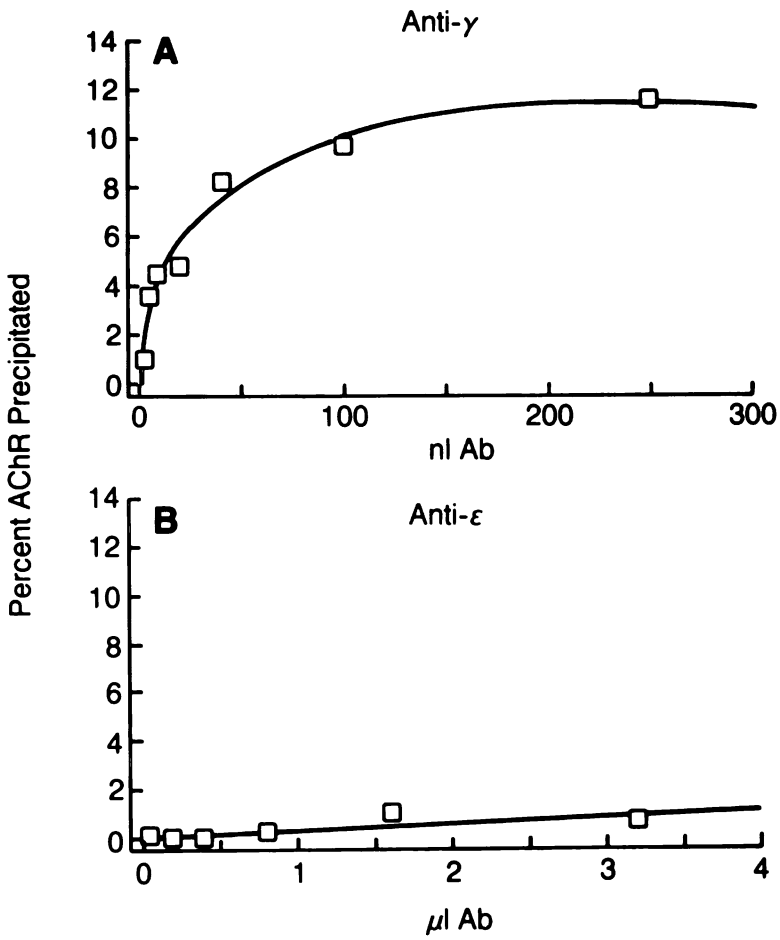


Figure 4-4. Immunoprecipitation of AChRs from endplate-rich and endplate-free regions of denervated rat muscle by anti-ε 360 antibodies. Crude extract of endplate-rich (□) or endplate-free (▨) regions of rat diaphragms that had been denervated for 10 days was incubated with ^{125}I-α-BuTx. The complex formed was then precipitated with 3 μl of anti-ε 360 antibodies or equivalent amount of normal rabbit IgG as described in Materials and Methods. The amount of receptor precipitated was expressed as a percentage of total AChR, which was 435 fmol. Background precipitation in the absence of first antibody was subtracted.

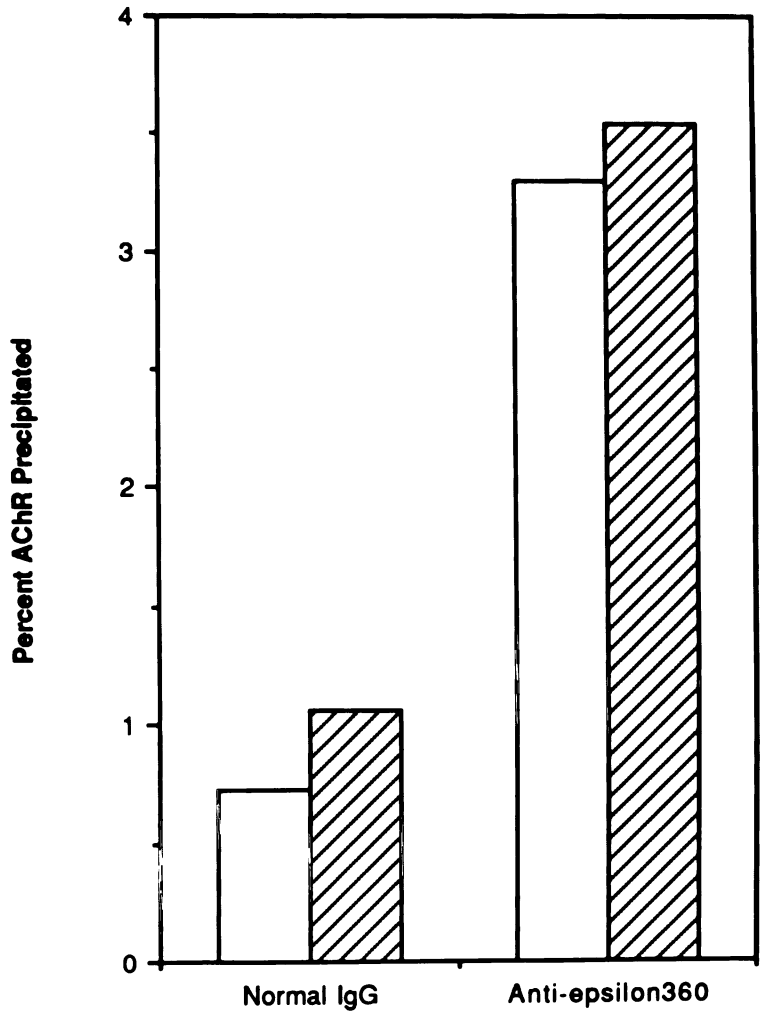

Percent AChR Precipitated
4
3
2
1
0
Normal IgG
Anti-epsilon360

Figure 4-5. Developmental changes of endplate AChRs. Cryostat sections (4-6 μm) of diaphragms taken from postnatal day 4, 9, and 16 and from adult rats were incubated with anti-γ 485 or anti-ε 360 antibodies, followed by fluorescein-conjugated second antibody and rhodamine-labeled α-BuTx. Endplates were then photographed with fluorescein or rhodamine optics. For anti-γ 485 antibody staining, the sections were pretreated with an alkaline solution for 3 min (as described in Materials and Methods). Such pretreatment did not change the pattern of staining by anti-ε 360 antibodies.

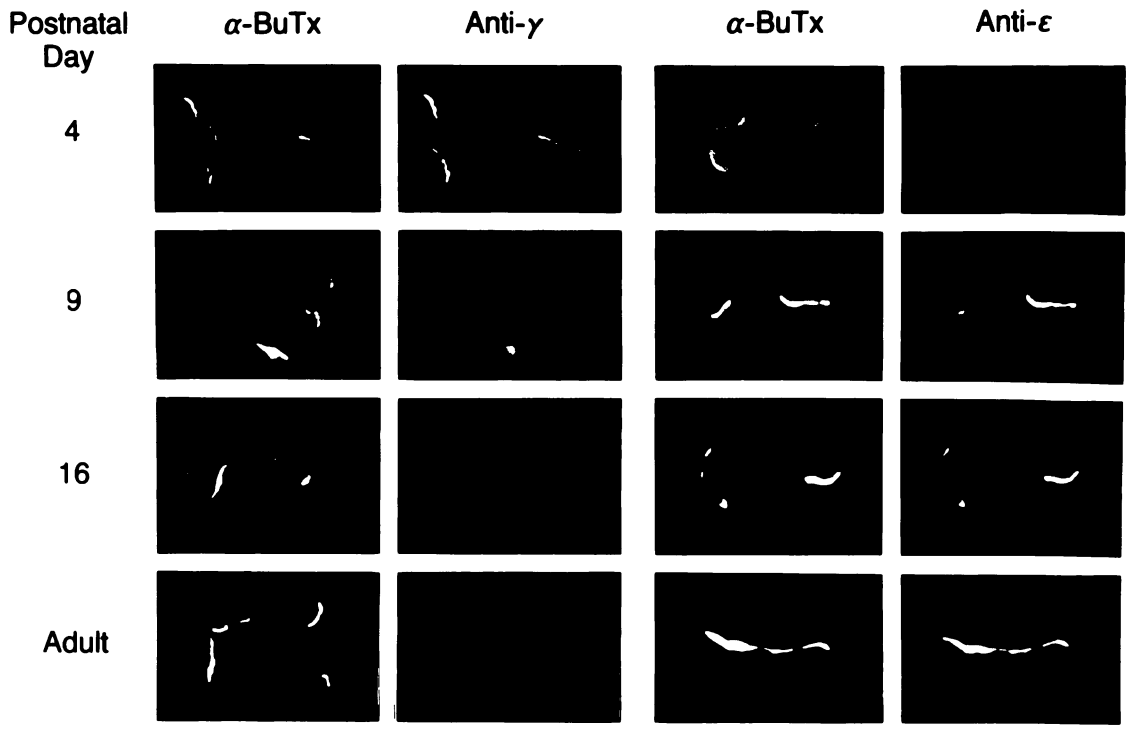

Postnatal Day
α-BuTx
Anti-γ
α-BuTx
Anti-ε
4
9
16
Adult

Figure 4-6. Immunofluorescent staining of rat endplates with anti-δ antibodies. Diaphragm sections with (pH 11.0) or without (pH 7.4) pretreatment with an alkaline solution were incubated with anti-δ 486 antibodies as described in Materials and Methods. Endplates were identified by rhodamine-labeled α-BuTx. Only the staining by the antibody is shown here.

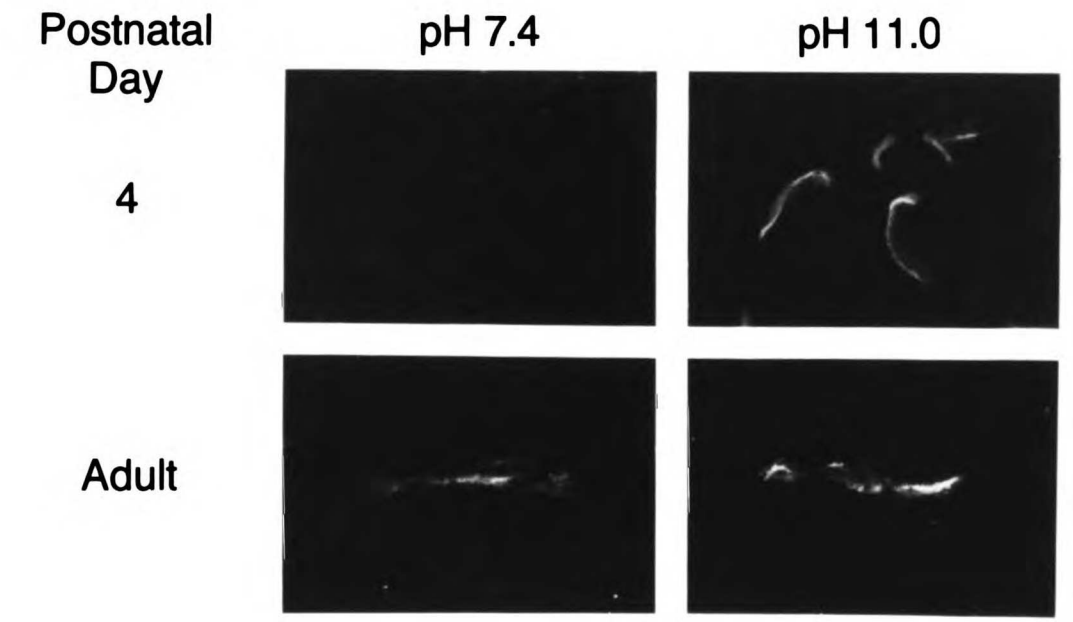
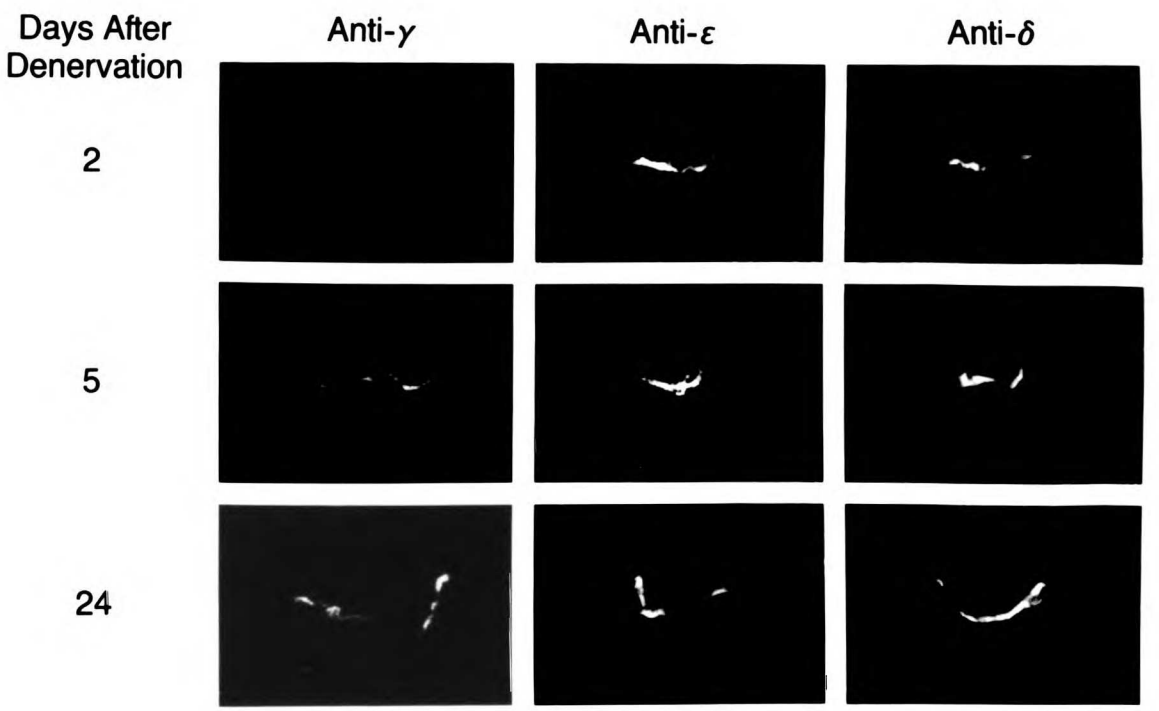

Postnatal Day
pH 7.4
pH 11.0
4
Adult

Figure 4-7. Immunological changes of AChRs at denervated endplates. Cryostat sections of rat diaphragms that had been denervated for 2, 5, and 24 days were pretreated with (Anti-γ) or without (Anti-ε and Anti-δ) an alkaline solution and were then stained with anti-γ 485, anti-ε 360, or anti-δ 486 antibodies as described in Materials and Methods. Only the antibody staining is shown here.


Days After Denervation
Anti-γ
Anti-ε
Anti-δ
2
5
24

Chapter 5

Characterization of Acetylcholine Receptor Subunits in Developing and in Denervated Mammalian Muscle

Abstract

We have used subunit-specific antibodies to identify and to characterize partially the α, β, γ, and δ subunits of rat skeletal muscle acetylcholine receptor (AChR) on immunoblots. The α subunit of rat muscle is a single band of 42 kDa, while the β subunit has an apparent molecular mass of 48 kDa. Both α and β subunits are glycosylated and contain one or more N-linked oligosaccharide chains that are sensitive to endoglycosidase H digestion. The γ and δ subunits, on the other hand, each appear as doublets on immunoblots, with apparent molecular masses of 52 kDa (γ), 48 kDa (γ') and 58 kDa (δ), 53 kDa (δ'), respectively. In each case, the two bands are structurally related and the lower band is probably the partial degradation product of the corresponding upper band. Each of the four γ and δ polypeptides is N-glycosylated and contains both endoglycosidase H-sensitive and endoglycosidase H-resistant oligosaccharides. When the AChRs purified from embryonic, neonatal, adult and denervated adult rat muscles were compared, no differences in the mobilities of α, β, or δ subunits on SDS gels were detected among them, either with or without endoglycosidase treatment. The γ subunits, which were present in AChRs purified from neonatal, embryonic or denervated rat muscles, were also identical; no γ subunit was detected, however, in AChRs of normal adult rat muscle.

Introduction

The nicotinic acetylcholine receptor (AChR) is a transmembrane glycoprotein that binds the neurotransmitter acetylcholine (ACh) and mediates synaptic transmission at the neuromuscular junction and at related synapses in the electric organs of *Torpedo* and *Electrophorus*. Because the *Torpedo* AChR can be easily obtained in large quantities, its structure and properties have been extensively studied. The oligomeric *Torpedo* AChR is composed of four different but highly related subunits that form a pentameric structure in which the subunits are arranged in the order $\alpha\beta\alpha\gamma\delta$ around a central cation-selective ion channel (McCarthy et al., 1986; Kubalek et al., 1987). Each of the subunit polypeptides has been isolated and identified, and its primary structure determined from cDNA clones (Numa et al., 1983; Patrick et al., 1983).

The mammalian muscle AChR shares biochemical and biophysical properties with the *Torpedo* AChR and antibodies to each of the subunits of the *Torpedo* AChR cross-react with individual subunits of muscle receptor (e.g. Conti-Tronconi et al., 1982; Brockes and Hall, 1975a; 1975b; Shorr et al., 1981; Lo et al., 1981; Tzartos et al., 1981; Sargent etal., 1984; Froehner etal., 1983; Balck et al., 1987; Einarson et al., 1982). Most importantly, cDNA clones corresponding to each of the four subunits of the *Torpedo* AChR have been obtained from mammalian muscle (Buonanno et al., 1986; Boulter et al., 1985; Kubo et al., 1985; Lapolla et al., 1984; Noda et al., 1983; Shibahara et al., 1985; Tanabe et al., 1984; Takai et al.,

1984; Yu et al., 1986). Injection of mRNAs derived from the four muscle cDNA clones into oocytes results in the production of functional receptor (Mishinia et al., 1986). A cDNA clone, corresponding to an additional subunit, ε, which is closely related to the γ subunit, has also been isolated from calf muscle (Takai et al., 1985).

These observations suggest that the overall structure of the AChR in mammalian muscle is similar to that of *Torpedo* AChR. The properties of the mammalian muscle AChR, however, change in several ways during development and maturation of the neuromuscular junction (Role and Schuetze, 1987; Salpeter, 1987), suggesting that the structure may also be altered. One of the changes, the postnatal alteration in the channel properties of rat and bovine muscle receptor, has recently been attributed to the postnatal replacement of γ subunit by ε (Mishina et al., 1986; Witzemann et al., 1987; Gu and Hall, 1988; see also Chapter 4).

To investigate further the molecular basis of changes during development, we have prepared specific antibodies against synthetic peptides with amino acid sequences that are unique to the γ and δ subunits, respectively, of the mouse muscle AChR (Gu and Hall, 1988; see also Chapter 4). We report here the use of these antibodies, along with subunit-specific monoclonal antibodies, to identify and to compare on protein blots each of the four subunits (α, β, γ and δ) of the AChR in developing and in denervated and normal adult rat muscle. In addition, their patterns of N-linked

glycosylation, determined by susceptibility to specific endoglycosidases, is described.

Materials and Methods

Antibodies. Four monoclonal antibodies (mAbs) and two subunit-specific antisera were used as probes to recognize specific subunits on immunoblots. All four monoclonal antibodies were raised against *Torpedo* AChR either in rats (mAbs 61, 124, generous gifts from Dr. Jon Lindstrom, The Salk Institute for Biological Studies, San Diego, CA) or in mice (mAbs 88B, 14-3-F7) as described in Tzartos et al. (1981); Gullick and Lindstrom (1983); Froehner et al. (1983) and Dowding and Hall (1987). mAbs 61 and 14-3-F7 recognize the α subunit (Black et al., 1987; Ratnam et al., 1986a, 1986b; Dowding and Hall, unpublished results). mAb 124 binds to the β subunit (Sargent at al., 1984; Black et al., 1987; Gullick and Lindstrom, 1983). mAb 88B, generously given to us by Dr. Stanley C. Froehner (Dartmouth Medical School, Hanover, NH), recognizes specifically the γ and δ subunits of *Torpedo* AChR and cross-reacts with cytoplasmic domains of mammalian muscle AChR (Froehner et al., 1983; Bloch and Froehner, 1987). The subunit-specific antibodies were obtained by affinity purification from corresponding rabbit antisera raised against synthetic peptides with subunit-specific sequences (Gu and Hall, 1988; see also Chapter 4). Anti-γ 485 antibodies recognize a peptide sequence corresponding to the amino acid residues 485-497 of the γ subunit of mouse muscle AChR and anti-δ 486 antibodies recognize a peptide sequence corresponding to

amino acid residues 486-496 of the mouse muscle AChR δ subunit. Both antibodies specifically immunoprecipitate their corresponding subunit polypeptides produced by *in vitro* translation and both recognize solubilized AChRs as well as AChRs *in situ* (Gu and Hall, 1988; see also Chapter 4).

Preparation of AChRs from Rat Muscles and from *Torpedo* Electric Organ. Acetylcholine receptors were partially purified from rat muscles or *Torpedo* electric organ using a procedure modified from that described previously (Brockes and Hall, 1975a, 1975b). Tissue obtained from normal or denervated lower leg muscle of adult rats or from neonatal or embryonic (ED 16-19) rats or from electric organ of *Torpedo californica* was homogenized in a Waring blender in 4 volumes of ice cold homogenization buffer A containing 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM N-ethylmaleimide (NEM), 1 mM sodium tetrathionate, 0.4 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM Tris-HCl, pH 7.4. The homogenate was centrifuged for 1.5 hr at 13,000 rpm in a GSA rotor and the pellet resuspended in 1 volume of homogenization buffer B containing 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1mM NEM, 1 mM sodium tetrathionate, 0.4 mM PMSF, 20 μg/ml leupeptin, 20 μg/ml pepstatin A, 1 mM benzamidine, 20 units/ml aprotinin, 50 mM Tris-HCl, pH 7.4. Triton X-100 was added to a final concentration of 1% and the homogenate extracted for 1 hr at 4 °C with occasional stirring. Insoluble material was then removed by centrifugation in an SS-34 rotor at 14,000 rpm for 45 minutes and the supernatant (S2) applied to an α-bungarotoxin-Sepharose (α-BuTx-Sepharose) column prepared as described in Brockes and Hall

(1975a) except that α-BuTx was used instead of α-cobratoxin. The resin was then washed with 10x bed volumes of TTEE buffer (50 mM Tris-HCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, pH 7.4), 10 volumes of the same buffer containing 1 M NaCl, and then again with 10 volumes of TTEE. For immunoblotting analysis, the receptor was eluted from the resin by resuspending and boiling the resin in SDS-PAGE sample buffer containing 3.5% (w/v) SDS, 10% (v/v) glycerol, 0.125 M Tris-HCl (pH 6.8), 5% (v/v) β-mercaptoethanol and 0.0025% (w/v) bromophenol blue for two minutes in a boiling water bath. For endoglycosidase digestion, AChRs were eluted from the Sepharose by resuspending and boiling in 0.6% SDS. Samples were stored at -20 °C until use. For the photoaffinity labeling experiment, the receptor in S2 was absorbed to α-cobratoxin coupled to Sepharose and eluted with 1.0 M carbamylcholine chloride in 10 mM Tris-HCl, 0.1% Tween 80, 1 mM EDTA, 1 mM EGTA, pH 7.4. Carbamylcholine chloride was then removed from the receptor preparation by dialysis against 4x 1 liter of the same buffer without carbamylcholine. The receptor preparation was kept on ice and used within one week. In some experiments, the extract (S2) was split into two parts. One part was processed as described above to isolate AChRs. The other was processed identically but was incubated with excess α-BuTx for 2 hours before application to the affinity column. This sample was used as a control for non-specific binding of proteins to the affinity column. The entire purification procedure lasted 5-6 hours.

Endoglycosidases H and F Digestion. Receptors purified as described above were diluted three-fold with H_2O and digested in a

mixture containing 0.2% SDS, 20 mM sodium citrate (pH 5.5), 5 mM EDTA and 10 mU/ml endoglycosidase H (endo H, Miles Laboratories, Inc., Naperville, IL) or 4 U/ml endoglycosidase F (endo F, New England Nulear, Boston, MA) or both. Digestion was carried out at 37 °C for 15-18 hours and was stopped by boiling in SDS-PAGE sample buffer for 2 minutes. Digestions were complete under the conditions described.

Photoaffinity Labeling of Muscle AChR Subunits. A photoaffinity derivative of α–BuTx (ANB-AI-α-BuTx) was prepared according to Nathanson and Hall (1980). One to 2 mg of ANB-AI (ethyl N-5-azido-2-nitrobenzoylaminoacetimidate·HCl, a generous gift from Dr. William S. Allison, University of California, San Diego, CA) was mixed with approximately 10 nmol of α-BuTx in 0.1 M Na_2CO_3, pH 9.0 under dim light and gently shaken in the dark for 12 hours at room temperature. The reaction mixture was then applied to a Bio-gel P-2 column and eluted with 10 mM Na/PO_4, pH 7.4 to separate the derivatized toxin from unreacted ANB-AI. The derivatized toxin was stored at -20 °C in the dark and used within three weeks.

To label the receptor, partially purified AChRs in 10 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 0.1% Tween 80, pH 7.4 were incubated with about 10 nM of derivatized α-BuTx with or without 100-fold excess of underivatized toxin for 2 hours at 37 °C in the dark. The reaction mixture was then transferred to a piece of parafilm floating in an ice-cold water bath and irradiated for 40 minutes

with a 250-Watt heat lamp. A 0.4 cm-thick glass plate was used as a short wavelength U.V. filter. The samples were then mixed with equal volumes of 2x concentrated SDS-PAGE sample buffer and subjected to electrophoresis and immunoblotting analysis as described below.

SDS-PAGE and Immunoblotting Analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the general procedure described previously (Laemmli, 1970), using phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14 kDa) as molecular weight standards (Bio-Rad, Richmond, CA). AChR subunits of partially purified receptors with or without prior endoglycosidase digestion were separated on 9% SDS-polyacrylamide gels. They were then electrophoretically transferred onto nitrocellulose membranes in a Hoefer transfer apparatus (model TE 50) in a buffer containing 20 mM Tris-base, 150 mM glycine, 20% (v/v) methanol. After quenching with 0.3% (v/v) Tween 20, 5% (w/v) hemoglobin in phosphate-buffered saline (PBS, 25 mM Na/PO_4, 144 mM NaCl, pH 7.4), the nitrocellulose was incubated with appropriately diluted primary antibody in the same buffer for 8-12 hours at room temperature, followed by ^{125}I-labeled second antibody for 2 hours and autoradiographed.

Peptide-mapping and 2-dimensional Gel Electrophoresis. Peptide-mapping of AChR subunits was carried out using a procedure

modified from Nathanson and Hall (1979). Partially purified AChRs were subjected to SDS-PAGE on a 0.75 mm-thick, 9% SDS-polyacrylamide slab gel as described above to separate the subunits. The strip containing the sample was then excised from the gel and soaked in 0.1% SDS, 0.125 M Tris-HCl, pH 6.8 for 10-15 minutes. It was then secured horizontally at the top of a second SDS-polyacrylamide gel with 1% agarose in SDS-PAGE sample buffer without bromophenol blue. The second gel was 1.5 mm thick and contained, from bottom to top, a 10-cm 15% separating gel, a 4-cm 5% stacking gel and a 0.5-cm 1% agarose gel containing 0.267 mg/ml *S. aureus* V8 protease (Miles Laboratories, Inc., Naperville, IL) in SDS-PAGE sample buffer without bromophenol blue. The gel was run at a constant current of 30 mA/gel till the dye front was about 0.5 cm away from the stacking/separating gel junction. Electrophoresis was interrupted for a period of 30 minutes to allow digestion and then continued at 30 mA/gel until the dye front passed the stacking/separating gel junction, at which time the current was increased to 60-80 mA/gel. Electrophoresis was stopped when the dye front reached the bottom and the gel was processed for immunoblotting as described above. Bromophenol blue (0.0002%, w/v) was added to the top electrode buffer reservoir at the beginning of electrophoresis.

Results

Identification of Mammalian Muscle AChR Subunits. We used subunit-specific antibodies to identify the subunits of AChRs

partially purified from denervated rat muscle, using *Torpedo* AChR as a reference. Lower leg muscle of rats whose sciatic nerve had been cut two weeks previously were used as the source of denervated muscle. AChRs were isolated from crude Triton X-100 extracts of muscle by affinity chromatography on an α-BuTx-Sepharose column. To minimize protease degradation, the receptor was eluted from the column by resuspending and boiling the resin directly in SDS-PAGE sample buffer. Non-specific binding of proteins to the resin was determined by preincubation of muscle extract with excess α-BuTx before application to the affinity column (control preparation), thus preventing the AChR from binding to the column. Any protein eluted from the column under these conditions therefore represents non-specific binding.

To identify each subunit of the receptor, partially purified AChRs or control preparations were analysed on a 9% polyacrylamide gel in SDS under reducing conditions. The proteins in the gel were then electrophoretically transferred to nitrocellulose membranes and probed with antibodies specific for α, β, γ or δ subunits as described below.

The α and β subunits. The α and β subunits of mammalian muscle AChR had previously been identified as single bands from cultured rat or mouse muscle cells either by a combination of metabolic radiolabeling and immunoprecipitation with subunit-specific monoclonal antibodies or by immunoblotting of affinity-purified AChRs with specific antibodies (e.g. Black et al., 1987; Smith et al.,

1987; Merlie et al., 1981; Merlie and Lindstrom, 1983). To identify these two AChR subunits in denervated rat muscle, we prepared immunoblots with AChRs partially purified from denervated rat lower leg muscle, using mAbs 14-3-F7 and 61 for the α subunit and mAb 124 for the β subunit as probes. As shown in Figure 5-1, both mAb 14-3-F7 and mAb 61 recognized a single band of the AChR prepared from denervated rat muscle. The band migrated at an apparent molecular mass of 42 kDa, similar to that of the α subunit of the *Torpedo* AChR (Figure 5-1a, lane 1). mAb 124, which recognizes the β subunit of the *Torpedo* AChR and cross-reacts with the corresponding subunit of the mammalian muscle AChR (Sargent et al., 1984; Black et al., 1987; Gullick and Lindstrom, 1983), also identified a single band from the receptor preparation, in this case with an apparent molecular mass of 48 kDa (Figure 5-1c). When immunoblots of control receptor preparations were tested, no bands were labeled by any of the 3 antibodies (Figure 5-1 a, b and c, lanes 3), demonstrating that the bands recognized by mAbs 14-3-F7 and 61 or by mAb 124 were specifically associated with the receptor. Incubation of the receptor preparation with mAb 61 followed by goat-anti-rat IgG coupled to Sepharose also specifically precipitated the band recognized by mAb 14-3-F7 (data not shown). Thus, in agreement with the results obtained with AChRs of cultured muscle cells, the α and β subunits of AChRs in denervated rat muscle each appear as a single band on SDS-polyacrylamide gels with apparent molecular masses of 42 kDa and 48 kDa, respectively.

The γ and δ subunits. We used two polyclonal anti-peptide antibodies and one monoclonal antibody to identify the γ and δ subunits. The two subunit-specific anti-peptide antibodies were affinity-purified from corresponding antisera raised against synthetic peptides with unique sequences of the γ or the δ subunit of mouse muscle AChR, respectively. Both antibody preparations were specific for their corresponding immunizing peptides and for their respective subunits produced in an *in vitro* translation system (Gu and Hall, 1988; see also Chapter 4). mAb 88B was prepared against *Torpedo* AChR and recognizes both γ and δ subunits of the *Torpedo* receptor on immunoblots (Froehner et al., 1983). It also binds to muscle AChRs and has been thought to recognize both γ and δ subunits as well (Bloch and Froehner, 1987).

When the two anti-peptide antibodies were used on immunoblots of AChRs purified from denervated rat muscle, they recognized distinct pairs of bands. As shown in Figure 5-1e and f, anti-γ 485 antibodies recognized two bands with apparent molecular masses of 52 kDa and 48 kDa, refered to here as γ and γ' bands, respectively. Anti-δ 486 antibodies, on the other hand, recognized two bands with apparent molecular masses of 58 kDa (δ) and 53 kDa (δ'), respectively. In both cases, the bands identified by these antibodies were specifically eliminated in control preparations (Figure 5-1e and f, lanes 3), suggesting that they were associated with the AChR. Neither antibody preparation labeled bands in a *Torpedo* AChR preparation (Figure 5-1e and f, lanes 1), consistent with immunohistochemical results in which these two antibodies

were inactive in recognizing *Torpedo* AChR (Gu and Hall, unpublished results).

When mAb 88B was used as the probe, three bands were specifically labeled (Figure 5-1d and g). The upper band has a molecular mass of 58 kDa, corresponding to the δ band recognized by anti-δ 486 antibodies. The middle band migrated at a molecular weight similar to that of the δ' band seen by the anti-δ 486 antibodies and the γ band seen by anti-γ 485 antibodies. A third band, which appears at a much lower intensity compared to the other two bands, migrated at the same place as the γ' band seen by anti-γ 485 antibodies (Figure 5-1g). Our interpretation of the patterns seen by these three antibodies is that the anti-γ 485 and anti-δ 486 antibodies recognize the γ and δ subunits, respectively, while mAb 88B recognizes both γ and δ subunits, although with a higher affinity for the δ subunit than for the γ subunit. The possibility that the γ' band is a product of cross-reaction of the anti-γ 485 antibodies with the β subunit and that the δ' band is a product of cross-reaction of the anti-δ 486 antibodies with the γ subunit is eliminated by the finding that the anti-δ 486 antibodies recognized two similar bands in AChRs purified from normal adult rat muscle, whereas anti-γ 485 antibodies did not detect any band at all (see below).

To determine if the two bands seen by anti-γ 485 antibodies are related, we performed a peptide mapping experiment in which the proteins were first separated on a regular 9% SDS-polyacrylamide gel (first dimension), then treated with *S. aureus* V8 protease, and

the resulting peptide fragments separated on a second gel (second dimension, 15%). The final gel was processed for immunoblotting with anti-γ 485 antibodies as the probe. As shown in Figure 5-2, *S. aureus* protease digestion of the γ and γ' bands recognized by the anti-γ 485 antibodies resulted in exactly the same pattern of peptide fragments for each band, indicating that the two bands are highly related in their structures. The simplest interpretation is that the lower band, γ', is the product of partial degradation of the corresponding upper band, γ. Because of the low amount of δ', we were unable to make an unambiguous comparison for the δ and δ' bands recognized by anti-δ 486 antibodies. For both γ and δ subunits, the ratio of the two bands varied from preparation to preparation, consistent with the idea that in both cases, one is derived from the other by proteolytic cleavage.

Photoaffinity Labeling of Muscle AChR Subunits. To confirm that the protein bands recognized by the antibodies on immunoblots are associated with the receptor, we have combined the immunoblotting analysis with a photoaffinity labeling procedure that was used to label receptor subunits in membrane-associated AChRs *in situ* (Nathanson and Hall, 1979). Partially purified AChRs from denervated rat muscle were incubated with the ANB-AI derivative of α-BuTx for 2 hours. Covalent cross-linking of the derivatized toxin with receptor subunits was initiated by irradiation. The sample was then subjected to SDS-PAGE and immunoblotting analysis as described in Materials and Methods. As shown in Figure 5-3a, the β band labeled by mAb 124 was shifted to

a higher position on the gel by this procedure. The δ subunit, as detected by mAb 88B, was shifted in a similar way (Figure 5-3b). The increase in the apparent molecular weight of the bands seen by mAbs 124 and 88B most likely represents the covalent attachment of α–BuTx to each polypeptide through the photo-activable reagent ANB-AI, as it was completely blocked by excess amount of underivatized toxin (Figure 5-3a and b, lanes 3). In addition, when iodinated toxin was used in these experiments, the only radiolabeled bands detected were identical in mobilities to the shifted bands seen in the immunoblots (data not shown). These results indicate that the bands recognized by mAbs 124 and 88B are in the close vicinity of α-BuTx-binding sites that are carried by each of the two α subunits of the receptor (Gershoni et al., 1983) and are therefore likely to be part of the AChR. The α and γ subunits were not labeled in these experiments. A similar pattern of subunit labeling was obtained with solubilized AChRs of the electric organ of *Torpedo* or normal adult rat muscle under the conditions used (data not shown).

N-linked Glycosylation of Muscle AChR Subunits. We examined the patterns of N-linked glycosylation of mammalian muscle AChR subunits by treatment of partially purified receptors with endoglycosidase H (endo H) or endoglycosidase F (endo F), followed by SDS-PAGE and immunoblotting analysis for each subunit. Endo H recognizes N-linked oligosaccharides with 5 or more mannose residues (high-mannose oligosaccharides) and cleaves the chitobiosyl unit (Tarentino et al., 1978). Endo F, on the other hand,

recognizes all types of N-linked oligosaccharides and cleaves at the site of the Asn-GlcNAc linkage (Elder et al., 1982).

When the AChRs purified from denervated rat lower leg muscle were treated with endo H, the band seen by mAbs 14-3-F7 and 61 was shifted down by about 2 kDa (Figure 5-4a, lane 2), corresponding to the cleavage of one or more high-mannose oligosaccharides. Endo F digestion resulted in a similar change in the apparent molecular weight (Figure 5-4a, lane 3). These results, taken together, indicate that the α subunit of the rat muscle AChR contains only high-mannose oligosaccharide moieties. Similar results were obtained for the β subunit, except that in this case the product of endo F digestion had a slightly higher apparent molecular weight than the product of endo H digestion (Figure 5-4b, lanes 2 and 3, see also Figure 5-5). This difference is too small to be the result of incomplete digestion and is probably due to unknown factors that affect the mobility of the polypeptide on SDS-PAGE. Similar behavior after glycosidase treatment was observed with the β subunit of AChRs purified from C2 myotubes (Gu and Hall, unpublished results). Thus, the β subunit, as well as the α subunit, contains only endo H-sensitive, high-mannose N-linked oligosaccharides. In both cases, combined digestion of the receptor with endo H and F gave no further cleavage than did endo H or endo F alone, suggesting that the digestion was complete under the conditions used.

When the γ and δ subunits were analyzed with endo H and endo F, different results were obtained. As shown in Figure 5-4c and d, endo H digestion changed the mobilities of both bands of either γ or δ subunit by 3-4 kDa, corresponding to the trimming of 1-2 oligosaccharide chains from each polypeptide chain. Endo F digestion, on the other hand, resulted in a larger shift in their mobilities. Thus, endo F treatment resulted in a downward shift of both γ and γ' bands by about 6 kDa, corresponding to the trimming of 2-3 oligosaccharide chains. The same treatment resulted in a downward shift of the δ and δ' bands by 6-7 kDa, corresponding to the trimming of 3-4 oligosaccharide chains. For both endo H and endo F digestion, neither prolonged incubation nor increased amount of enzyme gave any further products, suggesting that the reaction was complete in both cases. Combined digestion of both enzymes also did not give any further digestion than did digestion with endo F alone. Thus, our results indicate that, as in *Torpedo* AChRs (Nomoto et al., 1986), the α and β subunits of rat muscle AChR contain only high-mannose oligosaccharides sensitive to endo H digestion, while the γ and δ subunits contain both endo H-sensitive, high-mannose and endo H-resistant, complex oligosaccharides.

Developmental Changes of Mammalian Muscle AChR Subunits. In order to study the subunit structure of AChRs in developing and in denervated rat muscle, AChRs were partially purified from embryonic (ED 16-19), neonatal, adult and denervated adult rat muscles. Each receptor preparation was then analysed for each of the subunits on immunoblots, both with and without prior endo H and

endo F digestion. As shown in Figure 5-5, mAb 14-3-F7 detected a band of about 42 kDa and endo H or endo F digestion resulted in a similar shift in the mobilities of the band in all four preparations. Similar results were obtained for the β and δ subunits, as detected by mAb 124 and anti-δ 486 antibodies, respectively. These results thus indicate that the α, β, and δ subunits undergo little or no change either in their peptide backbones or in their patterns of N-linked glycosylation during development or after denervation.

The γ subunit, detected by anti-γ 485 antibodies, on the other hand, undergoes a dramatic change. As shown in Figure 5-5c, when the receptors purified from embryonic, neonatal or denervated adult rat muscle were probed with the anti-γ 485 antibodies on immunoblots, two bands (γ and γ') were recognized from each preparation. No difference could be detected in the mobilities of the two bands in the three AChR preparations, either with or without endo H or endo F digestion. When AChRs purified from normal adult rat muscle were used, however, no band was detected by this antibody (Figure 5-5c, lanes 2, 6 and10). The absence of γ subunit was not due to increased proteolytic activities or to other factors that might prevent its detection in normal adult muscle. Thus AChRs purified from a mixture of normal and denervated muscles have γ subunits that are indistinguishable from that found in AChRs prepared from denervated muscle alone (Figure 5-6). Our results thus demonstrate that the γ subunit, or at least the determinant(s) recognized by anti-γ 485 antibodies on the subunit disappear during development.

Discussion

These experiments had three objectives: 1) to identify the subunits of the mammalian muscle AChR by immunoblotting; 2) to characterize their susceptibility to glycosidases; and 3) to compare the properties of the AChR subunits from developing, adult and denervated muscles.

We were successful in identifying four of the subunits, α, β, γ and δ. The patterns of staining that we observed were similar to those seen previously for rat AChR by Einarson et al. (1982). α and β subunits were identified using subunit-specific monoclonal antibodies raised against *Torpedo* AChR. In accord with previous studies of mammalian muscle AChR (Black et al., 1987; Einarson et al., 1982; Froehner et al., 1977; Merlie et al., 1982), our experiments show that these subunits from rat skeletal muscle occur as single bands on immunoblots with apparent molecular masses of 42 KDa and 48 KDa, respectively.

The pattern of γ and δ subunits on immunoblots was more complex. Both appear as doublets when identified by antisera raised against synthetic peptides corresponding to subunit-specific sequences. In each case both bands were asssociated with the AChR since they were not present in preparations from which the AChR had been specifically excluded by preincubation of the muscle extract with uncoupled α-BuTx before application to the affinity

column. The multiple bands also did not arise from cross-reactivity of antibodies to different subunits. The position of each band and its susceptibility to glycosidase treatment eliminates most of the possible cross-reactions involving different subunits. The remaining posssibility, that the larger γ subunit (γ) and the smaller δ subunit (δ') are the same, is eliminated by the results obtained with adult muscle. In that case both γ subunits are absent, and both δ subunits are present. We conclude that there are two forms of γ subunit and two forms of δ subunit that are present in our receptor preparations.

The differences in apparent molecular mass between the two bands, which is 3-4 kDa for the γ subunit and 4-5 kDa for the δ subunit, persist after the removal of N-linked oligosaccharides with endoglycosidase F, suggesting that they arise either from differences in the peptide backbone or from differences in covalent modifications other than N-linked glycosylation. One possibility is that the lower band in each case is derived from the upper band by partial protease degradation. Both γ and δ subunits of muscle AChRs have been previously shown to be highly susceptible to protease degradation (Einarson et al. 1982; Lindstrom et al., 1980). A cleavage that removes about 30-40 amino acid residues from either the N-terminal or C-terminal of the subunit polypeptides would result in a band that is similar to the lower bands in each case. Since our antibodies were directed against peptide sequences at the C-terminal of each subunit, the C-terminal must be intact. Both bands in each case seem to contain endo H-sensitive as well as endo

H-resistant forms of N-linked oligosaccarides, suggesting that the N-linked glycosylation sites were also intact. In both mouse and calf muscle AChRs, the first potential site for N-linked glycosylation is located near the N-terminus at positions 30 and 76 in the γ and δ subunits, respectively (Kubo et al., 1985; Lapolla et al., 1984; Takai et al., 1984; Yu et al., 1986). The human γ subunit has also its first potential N-glycosylation site at residue 30 (Shibahara et al., 1985). If the AChRs in rat muscle share the same pattern with mouse, calf or human AChRs, a cleavage around amino acid residue 30 could result in a partial degradation product that has all the properties discussed for the lower bands seen by the antibodies, i.e. a band with reduced molecular weight and an intact C-terminal and all the potential N-linked glycosylation sites. Our results with partial protease digestion and peptide mapping indicate that at least in the case of the γ subunit, the two bands are highly related in their structures, supporting the above hypothesis. An alternative explanation of the result is that the two bands in each case arise from differential splicing of the messenger RNAs. This seems unlikely, however, as Northern blot analyses have not detected a second species of RNA for either γ or δ subunits in rat or bovine muscles (Mishina et al., 1986; Witzemann et al., 1987). The only example of multiplicity of mRNAs for AChR subunits was reported in mouse fibroblast transfected with *Torpedo* AChR cDNAs (Claudio et al., 1987). Thus, the most likely possibility is that one band is the partial degradation product of the other.

We were unsuccessful in identifying a protein band corresponding to the ε subunit of the mammalian AChR. We have developed antibodies against a peptide corresponding to the bovine ε sequence that recognizes AChRs from adult, but not embryonic rat muscle, when tested by immunoprecipitation or by immunocytochemistry (Gu and Hall, 1988; see also Chapter 4). Unfortunately we have not been able to detect a band on immunoblots that is specifically labeled by these antibodies. Our lack of success may be due to unknown structural alterations which occur during denaturation and SDS-PAGE of the receptor, or could result from susceptibility of the subunit to proteolysis.

The second aim of the experiments was to examine the patterns of N-linked glycosylation. The susceptibility of the α, β, γ and δ subunits to endoglycosidases H and F reveals difference in the patterns of N-linked glycosylation between the various subunits. The α and β subunits apparently contain one or more N-linked oligosaccharides that are sensitive to endo H and therefore are of the high mannose type. This is consistent with the presence of a single potential site for N-linked glycosylation in the deduced amino acid sequence of mammalian α and β subunits (Boulter et al., 1985; Buonanno et al., 1986; Noda et al., 1983; Tanabe et al., 1984), and also consistent with results obtained for the α subunit from *Torpedo* electric organ and from cultured mammalian muscle cells (Nomoto et al., 1986; Merlie et al., 1982).

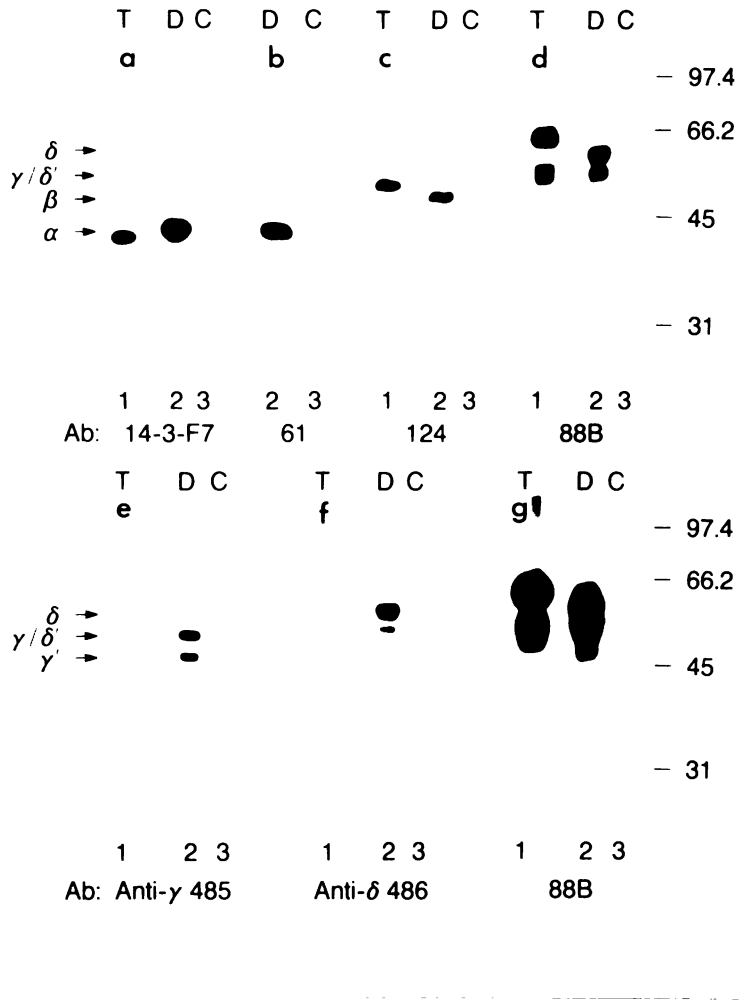
The γ and δ subunits, in contrast, contain both endo H-sensitive and endo-H resistant forms of N-linked oligosaccharides. Thus endo H digestion resulted in only a partly deglycosylated product compared to endo F digestion. In each of the cloned mammalian γ subunits, there are four potential N-glycosylation sites at positions 30, 141, 306 and 354, of which two are extracellular (positions 30 and 141) according to the postulated models of polypeptide folding (Shibahara et al., 1985; Takai et al., 1984; Yu et al., 1986). For the mouse δ subunit, there are three possible extracellular sites for N-glycosylation at positions 76, 143 and 169 (Lapolla et al., 1984). Although it is difficult to infer the numbers of N-linked oligosaccharide chains from changes in the apparent molecular weight upon endoglycosidase digestion, our results are consistent with the idea that both γ and δ subunits have multiple N-linked oligosaccharide chains, including both high-mannose and complex types. Although γ and δ subunits of the mammalian muscle AChR have not been previously analyzed, these results are consistent with those found by direct analysis of the *Torpedo* AChR (Nomoto et al., 1986).

Our final objective was to use immunoblots and glycosidase treatment to compare the AChRs of embryonic, neonatal, adult and denervated adult muscle. In the case of α, β and δ subunits, no differences were found, either in the mobilitites of the subunits on SDS gels or in their susceptibilities to endoglycosidases H and F. The γ subunit was present in embryonic, neonatal and denervated muscles where its properties were identical in each case. It was

undetectable, however, in adult muscle. This result is consistent with immunoprecipitation and immunohistochemical results using this same antibody preparation in which γ is also not detected in adult muscle (Gu and Hall, 1988; see also Chapter 4). Although we can not rule out the possibility of the presence in adult muscle of an altered γ subunit that lacks the C-terminal peptide sequence recognized by our antibodies, our results are consistent with the experiments of Mishina et al, (1986) and Witzemann et al. (1987), showing that in calf and rat muscle γ subunit mRNA is expressed only in early developmental stages.

During muscle development AChRs undergo several changes in their properties. One of these, the change in channel properties, is clearly associated with a change in subunit structure in which γ subunit is replaced by ε (Mishina et al., 1986; Witzemann et al., 1987; Gu and Hall, 1988; see also Chapter 4). Our results provide no evidence for other structural changes in the AChR that might accompany AChR clustering or the change in metabolic turnover time. These are thus presumably associated either with changes that are extrinsic to the AChR, or with changes in structure that are too subtle to be detected by our methods.

Figure 5-1. Identification of rat muscle AChR subunits. AChRs partially purified from the electric organ of *Torpedo californica* (T, lanes 1) or denervated rat lower leg muscle (D, lanes 2) on α-BuTx-Sepharose affinity column were subjected to SDS-PAGE on a 9% gel under reducing conditions. The proteins in the gel were then transferred onto nitrocellulose membranes and probed with mAb 14-3-F7 (panel a), mAb 61 (panel b), mAb 124 (panel c), mAb 88B (panels d and g), anti-γ 485 (panel e) or anti-δ 486 (panel f) antibodies, followed by iodinated second antibody as described in Materials and Methods. Lane 3 in each panel (C) is the same as lane 2 (D) except that the AChRs were specifically excluded from the preparation by incubating muscle extract with an excess of uncoupled α-BuTx before application to the affinity column (control preparation). Panel g is a longer exposure of panel d to show the third band seen by the antibody. The numbers on the right correspond to molecular mass in kilodaltons.


T D C
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88B
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T D C
f
T D C
g
97.4
66.2
45
31
δ
γ / δ'
γ'
1 2 3
1 2 3
1 2 3
Ab: Anti-γ 485
Anti-δ 486
88B

Figure 5-2. Peptide-mapping of the γ subunit of rat muscle AChR. AChRs prepared from denervated rat lower leg muscle were subjected to SDS-PAGE on a 9% gel under reducing conditions (first dimension). The strip that contained the sample was then cut out and overlain onto a 15% gel containing a layer of agarose with 0.267 mg/ml *S. aureus* protease (second dimension) as described in Materials and Methods. After the completion of digestion and electrophoresis, the peptide fragments were transferred to a nitrocellulose membrane and probed with anti-γ 485 antibodies as described. The figure shown is an autoradiograph of the immunoblots that had been exposed for 48 hours with an intensifying screen. *Arrows* mark the positions of γ and γ' bands in the first dimensional gel.

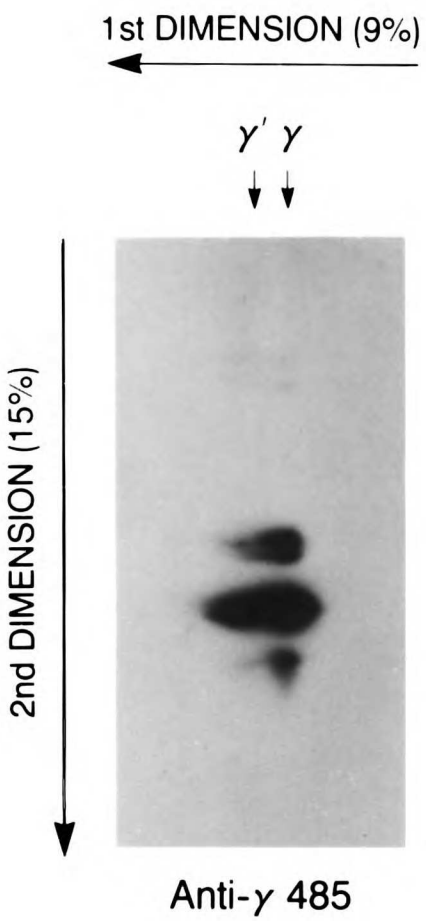

1st DIMENSION (9%)
γ' γ
2nd DIMENSION (15%)
Anti-γ 485

Figure 5-3. Photoaffinity labeling of solubilized rat muscle AChR. AChRs prepared from denervated rat muscle were incubated in the dark with (lanes 2 and 3) or without (lanes 1) 10 nM ANB-Al-α-BuTx in the presence (lanes 3) or absence (lanes 1 and 2) of 100-fold excess of underivatized toxin for 2 hours at 37°C . The samples were then transferred onto a piece of parafilm, irradiated and prepared for SDS-PAGE and immunoblotting analysis as described in Materials and Methods. In panel a, mAb 124 was used to detect the β subunit; in panel b, mAb 88B was used to identify the δ subunit. β-T and δ-T denote the β subunit-toxin and δ subunit-toxin complex, respectively.

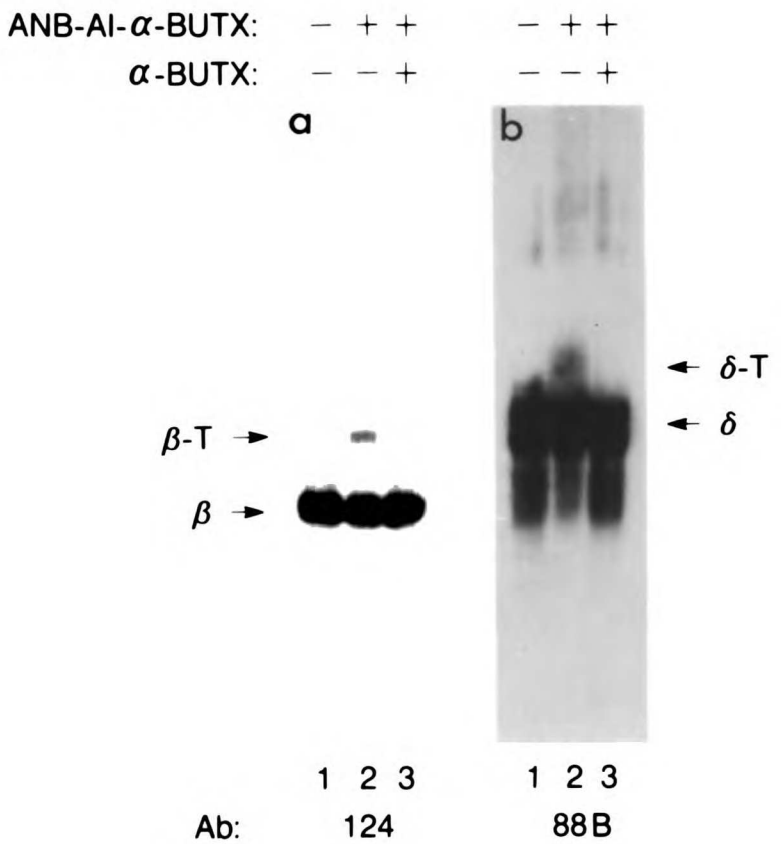

ANB-AI-α-BUTX: − + + − + +
α-BUTX: − − + − − +
a
b
β-T
β
δ-T
δ
1 2 3 1 2 3
Ab: 124 88B

Figure 5-4. N-linked glycosylation of rat muscle AChR subunits. AChRs prepared from denervated rat muscle were either mock-digested (lanes 1), digested with 10 mU/ml endoglycosidase H (lanes 2) or 4 U/ml endoglycosidase F (lanes 3) for 15 hours at 37 °C under conditions described in Materials and Methods. They were then subjected to SDS-PAGE and immunoblotted with mAb 14-3-F7 (panel a), mAb 124 (panel b), anti-γ 485 (panel c) or anti-δ 486 (panel d) antibodies to detect α, β, γ or δ subunit, respectively. The figure shows the autoradiographs of the blots. Numbers on the right corresponding to molecular mass in kilodaltons.

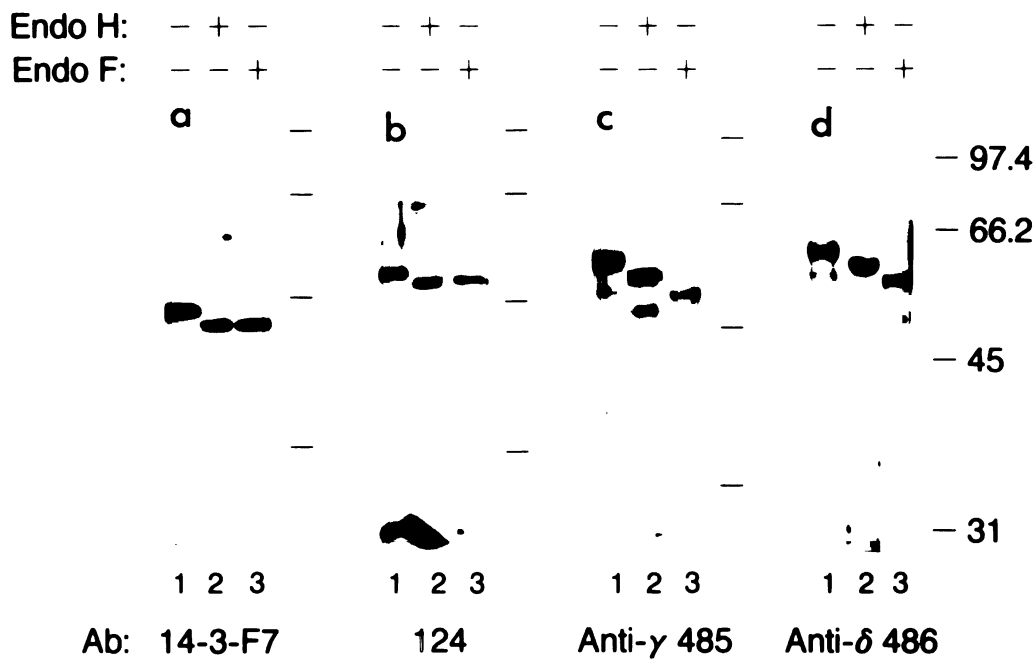

Endo H: − + −
Endo F: − − +
a
b
c
d
97.4
66.2
45
31
1 2 3
Ab: 14-3-F7
124
Anti-γ 485
Anti-δ 486

Figure 5-5. Developmental changes of rat muscle AChR subunits. AChRs were partially purified from muscles of embryonic (ED16-19) (E, lanes 4, 8 and 12), neonatal (N, lanes 3, 7 and 11), normal adult (A, lanes 2, 6 and 10) or denervated adult (D, lanes 1, 5 and 9) rats on α-BuTx-Sepharose affinity column, mock-digested (lanes 1-4), digested with 10 mU/ml endoglycosidase H (lanes 5-8) or 4 U/ml endoglycosidase F (lanes 9-12) for 18 hours at 37 °C as described in Materials and Methods. They were then subjected to SDS-PAGE and immunoblotting analysis. Antibodies used as probes were (panel a) mAb 14-3-F7 for α subunit; (panel b) mAb 124 for β subunit; (panel c) anti-γ 485 antibodies for γ subunit and (panel d) anti-δ 486 antibodies for δ subunit. The amount of samples loaded for the γ and δ subunits was twice the amount loaded for the α and β subunits. Note the absence of γ subunit in the AChRs prepared from normal adult rat muscle (panel c, lanes 2, 6 and 10). Molecular mass standards on the right are in kilodaltons.

DEVELOPMENTAL CHANGES OF RAT MUSCLE ACHR SUBUNITS

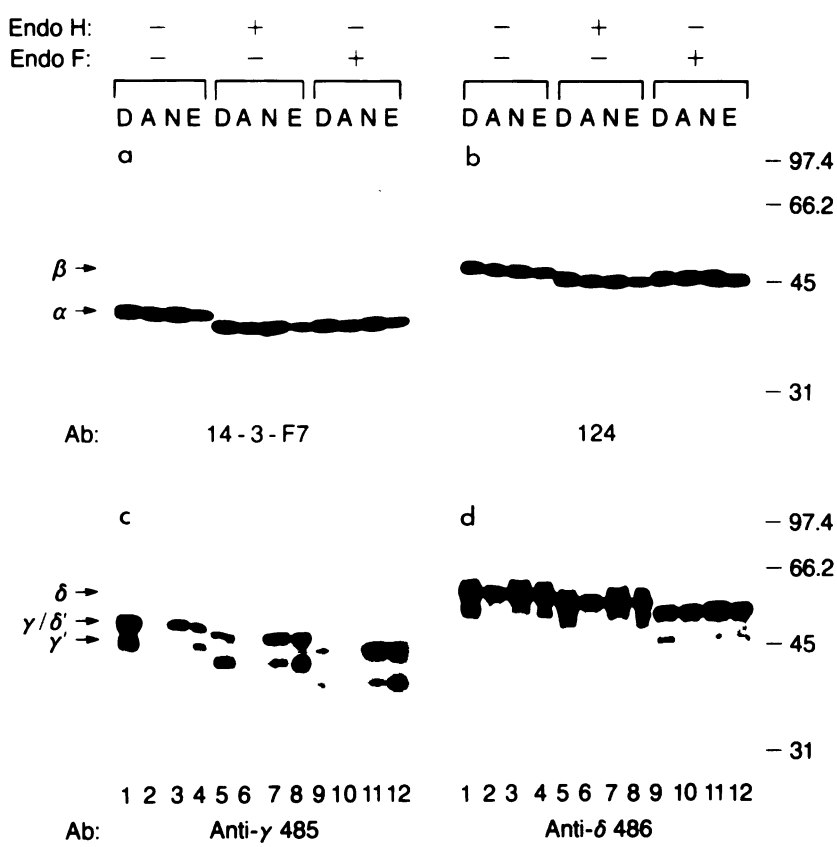
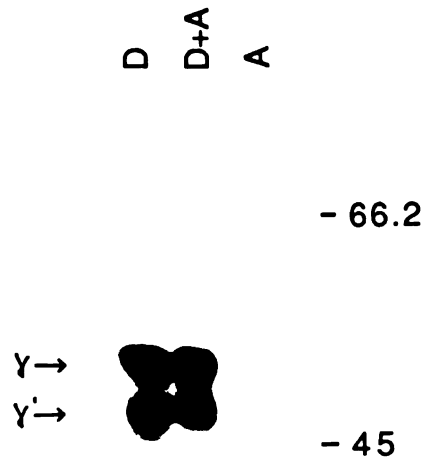


Figure 5-6. Absence of γ subunit in AChRs of normal adult rat muscle. AChRs partially purified from denervated (D), normal adult rat muscle (A), or mixed denervated and normal adult rat muscles were analyzed on 9% SDS-polyacrylamide gel and processed for immunoblotting as described in Materials and Methods, using anti-γ 485 antibodies as probes. Lane 1, AChRs obtained from denervated muscle alone (D); Lane 2, AChRs obtained from a mixture of denervated and normal adult muscles at 1 to 2 ratio in wet weight (D + A); Lane 3, AChRs from normal adult muscle alone (A). This experiment demonstrates that the absence of γ subunit on immunoblotts of AChRs of normal adult rat muscle is not due to increased proteolytic activity or to other factors in normal adult muscle that might have prevented its detection. Molecular mass standards on the right are in kilodaltons.


D
D+A
A
- 66.2
γ→
γ'→
- 45
1 2 3
Anti-γ 485

Chapter 6

Conclusion and Discussion

Using immunological methods, I have studied several aspects of the structure and function of the mammalian muscle nicotinic acetylcholine receptor. Firstly, I have studied the mechanisms underlying the partial inhibition of toxin binding by a serum from a patient with myasthenia gravis. Antibodies that block toxin binding to AChRs are common in myasthenic sera, and their titers correlate with the severity of the disease (Drachman et al., 1982). Thus, understanding the specificity of the antibodies and the mechanisms involved in the inhibition of toxin binding is not only of general interest but also of clinical importance. Taking advantage of the fact that the two toxin-binding sites on each receptor are intrinsically different in their affinities for some agonists and antagonists (Sine and Taylor, 1979, 1980, 1981; Neubig and Cohen, 1979; see also Chapter 2), I have used a mouse muscle cell line, C2, as a model system to study the specificity of the antibodies and have demonstrated that the antibodies block about one-half of the toxin binding by inhibiting the binding of toxin to one of the two toxin-binding sites on each receptor.

I have also studied the effect of this particular serum on the functional response of the receptor. Although the titers of the anti-toxin-binding site antibodies in myasthenic sera correlate with the severity of the disease, the mechanisms involved in the actions of the antibody is not well understood. Antibodies that block toxin binding presumably recognize a site near the cholinergic binding site, so it is plausible that such antibodies should also block channel function. Earlier studies have shown that anti-AChR antibodies may

exert their effects on endplate synaptic transmission by complement-mediated destruction of postsynaptic membranes or by increasing the rate of receptor degradation, thus decreasing the site density of the receptor at the endplate (reviewed by Drachman et al., 1981; Lindstrom and Engel, 1981). In Chapter 3, I have used carbamylcholine (CARB)-induced ^{22}Na uptake into C2 myotubes as a measure of the functional response of the receptor to cholinergic agonists. The results of these experiments demonstrate that the antibodies block the receptor function directly. The relation between the inhibition of toxin binding and the inhibition of ^{22}Na uptake by the antibodies suggests that blocking one of the two ligand-binding site is enough to impair the functional response completely. In collaboration with Drs. A. V. Maricq and S. Hestrin, I have also demonstrated that the antibodies inhibit the functional response of the receptor by inactivating individual receptors.

These studies thus demonstrate that antibodies in myasthenic sera may exert their effects on the postsynaptic membranes by inactivating individual receptors directly. Using the same procedure, Hall and colleagues (Hall et al., 1987) screened a number of myasthenic sera for their ability to block the binding of α-BuTx to the acetylcholine receptor and to inhibit the functional response of the receptor to cholinergic agonists in C2 cells. Of the 30 sera examined, 15 blocked toxin binding and each of them also blocked CARB-stimulated ^{22}Na uptake. On the other hand, only 3 of the 15 sera that do not block toxin binding showed inhibition of receptor function. Thus there is a very good correlation between the

inhibition of toxin binding and the inhibition of the functional response of the receptor by myasthenic sera. Although it was not possible to test the effects of these antibodies on the functional response of human AChRs, over 85% of the sera blocked toxin binding to human receptors.

The antibodies in the sera of myasthenic patients are of additional interest in that they usually contain antibodies that detect developmental changes in the structure of the receptor at the neuromuscular junction (see references in Chapter 2). In the particular serum studied, the antibodies recognize AChRs of embryonic muscles that exhibit the slow type of channel but not those in adult endplates that possess the fast channel type (Hall et al., 1985; Schuetze et al., 1985). The subunit specificity of this serum is unknown. But the specificity of the antibodies for embryonic AChRs resembles that of the anti-γ antibodies described in Chapters 4 and 5 of this thesis. Thus, the antibodies probably recognize the γ subunit itself or a determinant or determinants on the neighboring subunit(s) that is/are influenced by the γ subunit.

In Chapters 4 and 5, I have studied the changes in the structural properties of the receptor that underlie the developmental changes of its functional properties. By using subunit-specific synthetic peptides as antigens, I developed antibodies that are specific for the γ, δ, and ε subunits, respectively, of the mammalian muscle receptor. Immunocytochemical, immunoprecipitation, and immunoblotting experiments with these antibodies demonstrated that AChRs in

embryonic and denervated adult rat muscles contain γ but not ε subunit, whereas AChRs in normal adult endplates contain ε but not γ subunit. I have also shown that the AChRs change from anti-γ immunoreactive to anti-ε immunoreactive during the first and second postnatal weeks and that the distribution of γ- and ε-AChRs during development and after denervation correlates with the distribution of slow and fast channels, respectively.

The nicotinic acetylcholine receptor at the neuromuscular junction undergoes several changes in its functional properties during the formation and maturation of the endplate (Salpeter, 1987; Schuetze and Role, 1987). Evidence provided in this thesis and from other laboratories (Mishina et al., 1986; Witzemann et al., 1987) have demonstrated that one of the changes, the change in the channel properties of the receptor is associated with a change in the subunit composition of the receptor. While embryonic muscles express AChRs containing γ subunit (γ-AChRs) (see Chapters 4 and 5), which have a longer mean channel open time and a lower single channel conductance (Mishina et al., 1986), adult muscles express AChRs with ε subunit (ε-AChRs) (see Chapter 4), which have a higher single channel conductance and short mean channel open time (Mishina et al., 1986). In developing and in denervated adult muscles, both γ- and ε-AChRs are present, corresponding to the presence of both slow and fast type channels. One important question is how the expression of different types of receptor channels is regulated. Before considering possible mechanisms, it is useful to review some basic observations of the control of AChR expression.

Regulation of γ- and ε-AChR expression by the levels of specific subunit mRNAs.

Evidence exists that the expression of γ- and ε-AChR is controlled by the levels of specific subunit mRNAs (Mishina et al., 1986, Witzemann et al., 1987). Thus, in embryonic muscle, in which γ-AChRs with slow channels are expressed, high levels of γ subunit mRNA are also detected with little or no ε subunit mRNA. On the other hand, in adult muscles, in which fast channels containing ε subunit are dominant, high levels of ε subunit mRNA are found with an undetectable amount of γ subunit mRNA. In developing muscles in which both types of channels are present, both γ and ε mRNAs exist, corresponding to the presence of both γ- and ε-AChRs, at these endplates. Similarly, denervated muscles exhibit both low and high conductance channels and both γ- and ε-AChRs and molecular studies indicate the presence of both γ and ε mRNAs. Over 80% of the channel openings in denervated muscle are attributed to the low conductance channel type (Henderson et al., 1987), corresponding to an approximately 4 fold excess of γ-AChR over ε-AChR (see Chapter 4) and a 5 fold excess of γ mRNA over ε mRNA in the muscle (Witzemann et l., 1987).

The mechanisms involved in controlling the levels of specific subunit mRNAs remain to be explored. In principle, mRNA levels can be controlled either at the level of transcription or the degradation of a specific mRNA or a combination of both. Although there are

some evidence that favor the idea of transcriptional regulation (Shieh et al., 1987), distinction between these two mechanisms or other possible mechanisms needs further experimental evidence.

Neuronal control of AChR expression.

As discussed in Chapters 4 and 5, neuronal factors play important roles in the control of both the type and level of AChR expression in mammalian muscle.

Control of the level of AChR expression. There is evidence that both nerve-induced muscle activity and neural trophic factors are involved in regulating the level of AChR expression. In normal adult muscles, AChRs are detected only at the neuromuscular junction. Very low levels of AChR can be found outside the synaptic region. Denervation or inhibition of synaptic transmission increases the expression of both AChR mRNA levels and the rate of AChR synthesis (reviewed in Salpeter, 1987; Schuetze and Role, 1987). Similar effects can be obtained in cultured myotubes. Inhibition of spontaneous activity of the muscle with the voltage-dependent Na^+ channel blocker, TTX, increases both mRNA and AChR levels in the cell (Klarsfeld and Changeux, 1985). In contrast, chronical electrical stimulation of denervated muscles prevents or reverses the effect of denervation (Goldman et al., 1988). Thus, it seems that nerve suppresses the expression of AChR thoughout the muscle by nerve-induced muscle activity.

Although the mechanism of suppression of AChR expression by muscle activity is unknown, it does not seem to be specific for AChRs. Expression of other molecules in the muscle, including myosin light chains, N-CAM and the voltage-dependent Na^+ channel also seem to be regulated by innervation (Cooperman et al., 1987).

However, the nerve or the muscle must have a mechanism for sparing the endplate region from the general suppression of AChR expression exerted by nerve-induced muscle activity. One possibility is that local expression of AChR is induced through neuronal trophical factors. mRNAs coding for AChRs are concentrated in the endplate region (Merlie and Sane, 1985) and over 80% of the nuclei that express AChR mRNAs are localized underneath the nerve terminal (Fontaine et al., 1988). Another example of a molecule whose expression is under local control of nerve is the fast isoforms of myosin expressed in a slow muscle ectopically innervated by a fast nerve (Salviati et al., 1986).

Induction of ε-AChR expression by nerve. ε-AChR is not expressed to any significant extent in embryonic mammalian muscle cells until the later stages of development, when nerve-muscle contact is well established. The idea of nerve-dependent expression of ε-AChR is supported by the finding that early denervation of the muscle blocks the normal development of fast channels (Schuetze and Vicini, 1984). Furthermore, ectopic innervation of adult denervated muscle also induces expression of ε-AChR at the new endplates (Brenner et al., 1987).

Both muscle activity and neurotrophic influences seem to be involved in governing the expression of ε-AChR. Although early denervation can prevent or delay the development of ε-AChRs, chronical stimulation of the denervated muscle can reverse the effect of denervation (Brenner et al., 1987; Brenner, 1988). However, muscle activity alone is clearly not sufficient for the induction of ε-AChR. Spontaneously active muscle cells in culture in the absence of nerve do not express ε-AChR to any significant extent (Siegelbaum et al., 1984).

Whatever the mechanism is involved, the effect of the nerve on the expression of ε-AChR seems to be a permanent process. Evidence presented in this thesis demonstrates that denervated muscle continues to express ε-AChR at the endplate as well as in the extrajunctional membrane (Chapter 4). Electrophysiological studies also reveal that both types of channels are expressed in denervated muscles (Sakmann and Brenner,1983; Henderson et al., 1987; Brenner et al., 1983; 1987).

The effect of nerve on the expression of ε-AChR also seems to extent beyond the region of neuromuscular junction. In both normally innervated (Brehm and Kullberg, 1987) and denervated (Henderson et al., 1987) adult muscle, AChR channels of the fast type can be detected in both synaptic and non-synaptic membranes, corresponding to the presence of both ε-AChR (see Chapter 4) and ε subunit mRNA in these regions (Witzemann et al., 1987).

How could the nerve exert these effects? Any model of neuronal control of AChR expression in mammalian muscle has to take into account the regulation of the level of the AChRs expressed, the regulation of the types of AChR expressed, and the spatial and temporal distribution of the receptors in developing and in denervated muscles. One simple model that contain the following characteristics may account for all the aspects of neuronal regulation of AChR expression:

1. The expression of α, β, γ, and δ subunit is a default process that is turned on by the process of myogenesis in a tissue-specific and developmental stage-dependent manner;

2. Nerve exerts three different effects on AChR expression through nerve-muscle interactions: a). Nerve-induced muscle activity decreases or suppresses the expression of the receptor over the entire muscle fiber; b). Induction of ε expression in the innervated muscle by the nerve through both nerve-induced muscle activity and specific neurotrophic influences. This process is permanent, tissue-specific, and extends to the entire muscle fiber; and c). Induction of local expression of α, β, ε, and δ subunit genes in synaptic regions.

3. Denervation releases the suppression of AChR expression by nerve-induced muscle activity, thus increases the expression of both γ- and ε-AChRs in both junctional and extrajunctional regions.

According to such a model, the nerve influences the AChR expression through multiple channels, with some extending to the entire muscle fiber and others restricted to the endplate region, and with different subunit genes being regulated by different mechanisms. Although such a model seems to be able to explain most of the observations made so far, the detailed mechanisms involved remain to be experimentally explored.

Chapter 7

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