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The Nicotinic Acetylcholine Receptor: Structure and Autoimmune Pathology

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ABSTRACT: The nicotinic acetylcholine receptors (AChR) are presently the best-characterized neurotransmitter receptors. They are pentamers of homologous or identical subunits, symmetrically arranged to form a transmembrane cation channel. The AChR subunits form a family of homologous proteins, derived from a common ancestor. An autoimmune response to muscle AChR causes the disease myasthenia gravis. This review summarizes recent developments in the understanding of the AChR structure and its molecular recognition by the immune system in myasthenia.

KEY WORDS: ligand-gated ion channels, nicotinic receptor, ionotropic neurotransmitter receptors, myasthenia gravis.

I. INTRODUCTION

The unprecedented popularity enjoyed by the nicotinic acetylcholine receptor (AChR*) over the last 20 years among biochemists, immunologists, and molecular biologists has several reasons.

First, the AChR is by far the neurotransmitter receptor easiest to study because it can be easily purified in large amounts and in an active form from the electric tissue of *Torpedo* and *Electrophorous* fish and because of the existence of high-affinity polypeptide antagonists (several snake neurotoxins) that bind to different types of nicotinic receptors specifically and virtually irreversibly.

Second, in the 1970s, it was found that a syndrome very similar to the human disease myasthenia gravis can be induced in experimental animals by immunization against the AChR, and that the symptoms of human MG are indeed due to an autoimmune response to the AChR at the neuromuscular junction.

Third, during the 1980s, it became evident that all nicotinic receptors, of which a large variety is present in the nervous system, are structur-

* Abbreviations used: **AChR**, nicotinic acetycholine receptor; **MG**, myasthenia gravis; **EAMG**, experimental autoimmune MG; **EOM**, extrinsic ocular muscle; **MIR**, main immunogenic region; **α-BGT**, α-bungarotoxin; **α-NTX**, α-cobratoxin (α-najatoxin); **α-DTX**, α-dendrotoxin; **κ-BGT**, κ-bungarotoxin; **κ-FTX**, κ-flavitoxin; **NeSuTx**, neosurugarotoxin; **α-CnTx**, α-conotoxin; **STM**, scanning tunneling microscopy; **Carb**, carbamylcholine; **SDS**, sodium dodecyl sulfate; **MBTA**, 4-(*N*-malemido)benzyltri[^{3}H]-^{3}H-methylammonium; **BAC**, bromoacetylcholine; **DDF**, *p*-(dimethylamino)-benzenediazonium fluoroborate; **PCP**, phencyclidine; **Th**, T-helper; **MHC**, major histocompatibility complex.

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ally similar. They are symmetric or pseudo-symmetric pentamers of homologous subunits that have all derived from a common ancestor via multiple gene duplication.

Fourth, in more recent years, it was discovered that several other ligand-gated ion channels ("ionotropic" neurotransmitter receptors, for example, the $GABA_A$ and glycine receptors and the type 3 serotonin receptor) are members of the same protein family as the AChR. They appear to have a similar complex structure, being composed of homologous or identical subunits whose amino acid sequence is sufficiently similar to identify these functionally and pharmacologically disparate neurotransmitter receptors as members of the same protein family, originating from a common ancestor. This similarity allows the inference that the transmembrane folding, tertiary structure, topography of functionally important domains, mechanisms of activation, and ion gating may be alike in all members of this protein superfamily.

Therefore, the study of the AChR has gained momentum both because elucidation of its structure has implications for understanding how a human autoimmune response develops, and because it is reasonable to expect that the information acquired about the easily studied AChRs from fish electric organ will be relevant to the understanding of the structure and function of the other, less tractable members of the AChR ionotropic receptor superfamily.

Several review articles have been published in recent years on the structure, function, and autoimmune pathology of the AChR (e.g., Claudio, 1989; Stroud et al., 1990; Lindstrom et al., 1988; Levinson et al., 1987; Schönbeck et al., 1990).* We refer readers interested in exhaustive summaries of the field to those reviews. We focus the present review on more recent findings on the AChR structure and how they relate to its recognition by the human immune system in MG. We also review recent data on the unexpected variety of homologous AChRs found in the brain, which may be the structural basis for a cholinergic pharmacology in the brain whose variety and complexity had not been anticipated.

II. THE NICOTINIC ACETYLCHOLINE RECEPTOR AND MYASTHENIA GRAVIS: A BRIEF HISTORY

The great level of sophistication of our present knowledge on the structure and function of the AChR, and on the mechanisms of molecular recognition of this autoantigen in MG, were anticipated by the fact that some of the pioneering studies in physiology that led to the conclusion that neuronal transmission was mediated by a chemical were carried out on cholinergic systems (e.g., the frenic nerve/heart preparation). Those studies led to the conclusion that an essential component of neuronal transmission must be a "receptive substance" able to specifically recognize the chemical message released by the nerve and initiate the biochemical events leading to the response of the effector organ — in the case of the system used to demonstrate this principle, heart muscle contraction and a cholinergic receptor of muscarinic type.

Almost 90 years ago, Langley, based on the results of his experiments on the action of nicotine on avian muscle contraction, proposed that a "receptive substance..., combines with nicotine and curari and is not identical with the substance which contracts" (Langley, 1906; cited in Changeux, et al., 1984). Later, the morphological studies of neurons of Cajal (1911) revealed presynaptic terminals with distinct postsynaptic targets separated by a cleft. Since then, much

* Recent reviews published by our group on these matters include *Autoimmunity Against the Nicotinic Acetylcholine Receptor and the Presynaptic Calcium Channel at the Neuromuscular Junction*, by A. A. Manfredi, M. P. Protti, M. Bellone, L. Moiola and B. M. Conti-Tronconi, in *Ion Channels and Ion Metabolic and Endocrine Relationships in Biology and Clinical Medicine*, P. P. Foa and M. F. Walsh, Eds., Springer-Verlag, 1994, and *The Nicotinic Acetylcholine Receptor as a Model of a Superfamily of Ligand-Gated Ion Channel Proteins*, by K. E. McLane, S. J. M. Dunn, A. A. Manfredi, B. M. Conti-Tronconi and M. A. Raftery, in *Handbook for Protein and Peptide Design*, P. R. Carey, Ed., Academic Press, 1993. Portions of this article have been reprinted by permission of the publisher from *The Nicotinic Acetylcholine Receptor as a Model of a Superfamily of Ligand-Gated Ion Channel Proteins*, by K. E. McLane, S. J. M. Dunn, A. A. Manfredi, B. M. Conti-Tronconi and M. A. Raftery, in *Handbook for Protein and Peptide Design*, P. R. Carey, Ed., Copyright © 1995 by Academic Press, Inc.

knowledge has accumulated regarding the initial concepts of peripheral receptors stemming from studies of choline esters (Dale, 1914) as well as those of the CNS (Dale, 1935) that interact with the transmitter acetylcholine (Loewi, 1921). Our current understanding of neuronal signaling is due in large part to the ionic hypothesis (Hodgkin and Huxley, 1952; Hodgkin and Katz, 1949) that was put forward to explain how the resting membrane potential and the mechanisms underlying the action potential are generated. Shortly thereafter, Fatt and Katz (1951) showed that an extension of this hypothesis could also account for chemically induced synaptic transmission where receptor channels, such as the AChR, are gated by the binding of ligands to receptors rather than by voltage changes. They further showed that the difference between synaptic excitation and inhibition lay not in the nature of the transmitter, but rather was a function of the properties of the channels involved.

With further refinement of electrophysiological methods (see Neher, 1992; Sakmann, 1992), it became possible to study ion flow through a single channel protein molecule such as the AChR, which over a period of 1 to 10 ms transports ~2 × 10^4 cations per channel per microsecond, a value consistent with a channel rather than a carrier mechanism. Meanwhile, over the last 20 years, significant strides were made in the isolation and characterization of the protein molecule responsible for these effects. It became possible to show quantitatively that a single AChR molecule either in isolated membrane preparations (Moore and Raftery, 1980) or in reconstituted form, following purification to homogeneity in detergent solution and reconstituted into phospholipid vesicles (Wu et al., 1981) or planar lipid bilayers (Sakmann and Neher, 1983), transports cations as efficiently as shown for AChR channels *in vivo*.

MG has also been known for a long time, since a myasthenic patient was described by Thomas Willis in 1672. In 1892, Herman Hoppe, a pathologist from Cincinnati, reported the presence of a mediastinal tumor in myasthenic patients (Hoppe, 1892). In 1900, Campbell and Bramwell described in detail the clinical symptoms of MG, and in 1901, a relationship between MG and the thymus was first noted (Weigart, 1901). In 1913, a case of MG associated with hyperthyroidism (possibly Graves' disease, which is sometimes associated with MG) was reported that improved after thymectomy (Schumacher and Roth, 1913). In the 1930s, the unfolding of studies on chemical transmission at the neuromuscular junction (Dale and Feldberg, 1934), and the observation of similarities between the symptoms of MG and curare poisoning, suggested an impairment of neuromuscular transmission as the functional defect in MG and led Mary Walker to treat MG patients with anticholinesterase drugs (Walker, 1934). In the 1940s, thymectomy became an accepted procedure for treatment of MG (Blalock et al., 1941). An important step in the understanding of MG pathogenesis was the insight by Simpson in 1960 that MG could have an autoimmune origin (Simpson, 1960). In 1966, two groups independently reported the reactivity of MG serum immunoglobulins with skeletal muscle and thymic myoid cell determinants (Van De Velde and Friedman, 1966; Van Der Geld and Strauss, 1966). By the early 1970s, it was recognized that MG involved a defect in neuromuscular transmission, but, on the basis of electrophysiologic studies of MG muscles that revealed a reduced size of the miniature end plate potentials, a presynaptic abnormality in the synthesis/storage/release of ACh was believed to cause myasthenic symptoms (reviewed in Grob, 1987). Crucial for the understanding of MG pathogenesis was the discovery that rabbits immunized with purified AChR developed muscular weakness similar to that of MG patients (Patrick and Lindstrom, 1973), suggesting that human MG might have a similar pathogenesis. In 1975, Toyka and co-workers reported that MG symptoms could be transferred in mice by treatment with MG sera or their IgG fraction, thus providing a direct demonstration that antibodies are the effectors of MG symptoms. Those fundamental studies and others that followed (reviewed in Lindstrom et al., 1988) strongly suggested that MG symptoms could result from an autoimmune response against muscle AChR. Later, autoimmune AChR-specific antibodies and T cells were detected in the blood of MG patients, demonstrating that MG is indeed due to an autoimmune anti-AChR response (reviewed in Engel, 1984; Levinson et al., 1987; Lindstrom

et al., 1988; Schönbeck et al., 1990; Penn et al., 1993).

III. THE PRIMARY STRUCTURE OF AChRs IN PERIPHERAL AND NEURONAL TISSUES

A. Amino Acid Sequence of the AChR Subunits

The constituent subunits of AChRs from different species and tissues (muscle, electric organ, and neurons) have similar amino acid sequences, and they should therefore have common structural features.

Sequence characteristics shared by all AChR subunits include (1) a large putative extracellular N-terminal domain containing two cysteine residues separated by ~15 amino acids ("Cys-Cys loop"), (2) four putative transmembrane regions, designated M1 to M4, (3) conservation of a proline in the M1 segment, (4) an abundance of serine, threonine, and small aliphatic amino acids in the M2 segment, and (5) a long nonconserved region between M3 and M4 that is at least partly cytoplasmic (see also Section IV.C).

1. AChRs from Peripheral Tissues: Electric Organ, Skeletal Muscle, Extraocular Muscle, and Thymus. Isoforms of Muscle AChRs

Protein sequencing of the four purified *Torpedo* AChR subunits (α, β, γ, and δ) and subsequent cloning and sequencing of their encoding genes revealed a high degree (40 to 50%) of sequence identity among them (Raftery et al., 1980; Noda et al., 1982, 1983b, c; reviewed in Claudio, 1989; Stroud et al., 1990). Protein sequencing of the four subunits of AChRs from the electric tissue of *Torpedo* and *Electrophorus* — two distant species whose similar electric organs originated by convergent evolution — showed that both these AChRs are pentamers of homologous subunits sharing ~60% amino acid identity, in a stoichiometry of $\alpha_2\beta\gamma\delta$ (Raftery et al., 1980; Conti-Tronconi et al., 1982a). That was the first demonstration that AChRs from different species are members of the same protein family, and that the subunit structure and primary sequence of peripheral AChRs is highly conserved throughout evolution. This was verified by isolation and sequencing of the α-, β-, γ-, δ-, and ε-subunits — or cloning and sequencing of their encoding genes — of calf muscle AChRs (Conti-Tronconi et al., 1982b; Noda et al., 1983a; Tanabe et al., 1984; Takai et al., 1984, 1985; Kubo et al., 1985). The sequences of the constituent subunits of muscle AChRs from several species, including *Homo sapiens* and cobra, were determined subsequently (Noda et al., 1983a; Boulter et al., 1985; Barnard et al., 1986; Nef et al., 1986, 1988; Baldwin et al., 1988; Neumann et al., 1989), and they were all found to be highly conserved.

Mammalian muscle AChR exists in two developmentally regulated isoforms (reviewed in Schuetze, 1986). Embryonic muscle expresses AChRs composed of α-, β-, γ-, and δ-subunits. Upon innervation, the γ-subunit is substituted by a homologous ε-subunit (however, for an exception to this rule, see below), to yield adult AChR, and $\alpha_2\beta\varepsilon\delta$ oligomer (Mishina et al., 1986; Gu and Hall, 1988). This change in subunit composition alters the pharmacological and metabolic properties of the AChR and the conductance characteristics of the ion channel (Trautman, 1982; Hall et al., 1985; Schuetze, 1986; Mishina et al., 1986; Gu and Hall, 1988; Sakmann, 1992).

An exception to the rule that only denervated muscle expresses the γ-subunit has been demonstrated recently for extrinsic ocular muscles (EOM), which in the adult animal express both the γ- and the ε-subunit (Horton et al., 1993). EOM have two types of muscle fibers with different functional properties — tonic fibers specialized in sustained tonic contraction, and fast twitch fibers. Tonic and twitch fibers have morphologically different synapses (en plaque synapses on twitch fibers and en grappe synapses on tonic fibers) (reviewed in Kaminski et al., 1990). Tonic fibers, which have multiple synaptic terminals, have slow synaptic current kinetics, which would be well explained by the presence of "embryonic" AChRs. This may be functionally relevant for muscle fibers specialized in sustained tonic contraction, because the long channel-open time of embryonic AChR would cause a more uniform

spread of depolarization along the fibers, while its small conductance would make them more resistant to desensitization and better able to respond to prolonged nerve stimulation.

EOM are preferentially and sometimes uniquely affected by myasthenic symptoms: in 50 to 65% of MG patients, the initial symptoms involve only the EOM, and in 10 to 14% of MG patients, the symptoms remain restricted to the EOM for at least 2 years (ocular MG) (Osserman and Genkins, 1971; Drachman et al., 1987; Oda, 1993). This may be related to the presence of embryonic AChR because, as discussed in Section XIII, MG patients have antibodies and T-helper (Th) cells specific for embryonic AChR, and it is possible that an embryonic AChR-like protein expressed within the thymus (see below) is the primary antigen in MG. Furthermore, EOM endplates, and specifically those or multiterminal fibers, have unique antigenic properties, and MG patients suffering from ocular MG may have antibodies specific for epitopes unique to EOM. Such antibodies are pathogenic because they cause AChR loss in the EOM endplates of mice treated with IgG from these patients (Oda et al., 1981). The propensity of EOM to develop myasthenic symptoms may also be for other reasons, such as a smaller "safety factor" for neuromuscular transmission than skeletal muscle, because EOM are preferentially involved in congenital, nonautoimmune forms of myasthenia gravis (Engel, 1987).

Two isoforms of muscle α-subunits have been described in *Xenopus* (Hartman and Claudio, 1990) and in humans (Beeson et al., 1990). The human isoforms differ in that one of them contains an additional 22 amino acid residues, encoded by a separate exon just before the sequence region that contributes to formation of the main immunogenic region. Two isoforms of the mammalian muscle β-subunit also exist, resulting from alternative splicing of the mRNA (Goldman and Tanai, 1989). The functional significance of these different AChR subunit isoforms is not known.

The thymus contains a component(s) immunologically cross-reactive with muscle AChR (Aharonov et al., 1975; Ueno et al., 1980; Schleup et al., 1987; Kirchner et al., 1988) as well as binding sites for α-bungarotoxin (α-BGT), a snake toxin that specifically recognizes AChR from peripheral tissues (Engel et al., 1977; Kao and Drachman, 1977; Kawanami et al., 1988). The thymus α-BGT binding component has the subunit structure and physicochemical properties expected for a true AChR, and cross-reacts with antisera raised against *Torpedo* AChR (Kawanami et al., 1988). Subunit-specific antibodies demonstrated that the thymus AChR-like protein contains subunits immunologically related or identical to all those of embryonic muscle AChR, that is, α-, β-, γ-, and δ-subunits (Nelson and Conti-Tronconi, 1990). The presence of mRNA for AChR muscle α-, β-, γ-, and δ-subunits has been verified in normal thymus tissue as well as in hyperplastic MG thymuses by dot blot hybridization (Geuder et al., 1992). The occurrence of α-subunit transcripts was demonstrated in thymomas by southern blot hybridization (Muller-Hermelink et al., 1993). A large part of the primary sequence of the α-subunit expressed in mouse and human thymuses was determined. Consistently, the regions sequenced were identical to the muscle α1 subunit of that species. Human thymuses express both the α-subunit isoforms found in human muscle (Wheatlay et al., 1993).

The thymus may also express neuronal AChRs because it receives a rich parasympathetic innervation, which is important for the development and possibly the function of thymus (Williams and Felten, 1981; Felten et al., 1985; Bullock, 1987; Magni et al., 1987; Bullock and Pomerantz, 1984; Rozzman and Brooks, 1985). The presence of the neuronal α3 subunit in normal and hypertrophic thymuses from MG patients was demonstrated by PCR analyses and sequencing of thymus cDNA clones, northern blotting, and RNA protection assays (Mihovilovic et al., 1993). Curiously, a patient has been identified with MG and thymoma and also antibodies to the α3 subunit (Grando and Conti-Tronconi, unpublished results).

2. AChRs of Autonomic Ganglia and Central Nervous System: Multiple Subtypes of α- and β-Subunits

Several AChR subunits have been identified and sequenced from mammalian and nonmammalian neuronal tissues (Table 1). Neuronal

Table 1
Neuronal AChr Subunits Identified by Low-Stringency Hybridization

AChR subunit	Probe	Source	Ref.
α3	Mouse muscle α1 subunit	PC12 cell line (rat pheochromocytoma)	Boulter et al., 1986
α2, α4, α5, β2, β3	Rat α3 subunit	PC12 and rodent brain	Wada et al., 1988; Goldman et al., 1987; Boulter et al., 1990a; Deneris et al., 1988, 1989
β4, α6, β5[a]	Rat neuronal α and β subunits	Rodent brain	Duvoisin et al., 1989; Deneris et al., 1991
α2, α3, α4	Chicken muscle α1 subunit	Chicken brain and autonomic nervous system	Nef et al., 1988; Barnard et al., 1986; Ballivet et al., 1988; Schoepfer et al., 1988
α5, nα3[b]	Chick α3 gene cluster	Chicken brain	Couturier et al., 1990a
nα1, nα2[b]	Chicken muscle α1 subunit	Chicken brain and autonomic nervous system	Ballivet et al., 1988; Schoepfer et al., 1988; Couturier et al., 1990a; Deneris et al., 1991
α7 (or αBGTBPα1) α8 (or αBGTBPα2)	N terminus of an α-BGT-binding, 48-kDa subunit	Chicken brain	Conti-Tronconi et al., 1985; Schoepfer et al., 1990; Couturier et al., 1990b
GFα-3, GFnα-2, GFnα-3	*Torpedo* and rat muscle α1 and rodent α4 subunits	Common goldfish (*Carassius auratus*) brain	Cauley et al., 1989, 1990
ARD (β homolog), ALS, or Dα1 (α3 homolog)	Vertebrate AChR subunits	*Drosophila*	Schlob et al., 1988; Bossy et al., 1988
SAD or Dα2 Dα3	ALS-conserved M4 oligonucleotides	*Drosophila*	Schlob et al., 1988; Gundelfinger, 1992
αL1 or ARL2, αRL1	Chick β2 subunit	*Schistocerca gregaria*	Marshall et al., 1990; Hermsen et al., 1991; Gundelfinger, 1992

[a] α6, β5 sequences and characterization have not been reported to date.
[b] nα means non-α; also called structural αβ subunits.

From McLane, K. E., Dunn, S. J. M., Manfredi, A. A., Conti-Tronconi, B. M., and Raftery, M. A. The nicotinic acetylcholine receptor as a model of a superfamily of ligand gated ion channel proteins. **In:** *Handbook for Protein and Peptide Design,* Carey, P. R., Ed., Copyright © 1995 by Academic Press, Inc. With permission.

AChR subunits can be classified on the basis of the sequence homology among themselves and with the subunits of peripheral tissue AChRs, as α-subunits, which contain within their N-terminal extracellular segment a vicinal pair of cysteine residues, the hallmark of all AChR α-subunits, and β (or non-α)-subunits. Conventionally, muscle AChR subunits are indicated with the postscript 1 (α1, β1), and neuronal subunits with postscript numbers that indicate the order in which a particular subunit was identified and sequenced. Neuronal subunits from α2 to α8 and from β2 to

β5 have been described to date (Table 1). Subunits corresponding to the muscle γ-, ε-, and δ-subunits have not yet been described in neuronal systems. The amino acid sequences of different rodent AChR α- and β-subunits are 40 to 70% identical (Boulter et al., 1990a). It is not surprising that neuronal subunits are homologous to the muscle AChR sequences and to each other, because they were selected on the basis of homology. The number of different AChR subunits expressed in the brain, however, was not anticipated.

B. Functional Heterogeneity in AChR Subtypes Induced by Different Subunit Combinations

Functional diversity conferred by different combinations of subunits was first described for the AChRs of embryonic and adult mammalian muscle (Mishina et al., 1986) (see Section III.A.1). While muscle AChRs contain four different subunits, neuronal AChRs may contain only two subunits, α and β, or even one subunit only (α) (Goldman et al., 1987; Wada et al., 1988; Duvoisin et al., 1989; Couturier et al., 1990b; Bertrand et al., 1992). Coexpression of different neuronal α-subunits with either the neuronal β2 or β4 subunits in *Xenopus* oocytes results in acetylcholine (ACh)-gated cation channels with different conductance, open times, and burst kinetics (Papke and Heinemann, 1991). The different α-subunits expressed in the central and autonomic nervous systems endow cholinergic neuronal pathways with multiple response states of potential functional significance (Papke et al., 1989; Papke and Heinemann, 1991).

Different subunits of rodent neuronal AChRs confer differential pharmacology to AChRs expressed in *Xenopus* oocytes. The α-subunit subtype is important in determining the differential sensitivity of the resulting AChR complex to neurotoxins from invertebrates and snake venoms. Two classes of snake neurotoxins from *Bungarus multicinctus* and *B. flavus* venom distinguish AChR subtypes, that is, the α-neurotoxins, such as α-bungarotoxin (α-BGT), and the κ-neurotoxins, κ-bungarotoxin (κ-BGT) and κ-flavitoxin (κ-FTX) (Chiappinelli, 1985; Grant et al., 1988). κ-BGT (also referred to as toxin F [Loring et al., 1984], bungarotoxin 3.1 [Ravdin and Berg, 1979], and neuronal bungarotoxin [Lindstrom et al., 1987]) and α-BGT were initially regarded as specific antagonists of ganglionic AChR and muscle AChRs, respectively. Molecular genetic approaches, however, have revealed that this simple dichotomy does not hold.

α-BGT irreversibly blocks ($t_{1/2}$ > 200 h, [Blanchard et al, 1979]) AChRs formed by the subunit combinations α1β1γδ and α1β2γδ (Deneris et al., 1988). Many avian and rodent neuronal AChRs comprising different α/β subunit combinations — α2β2, α3β2, α4β2, and α3β4 — are insensitive to α-BGT (Deneris et al., 1988; Wada et al., 1988; Duvoisin et al., 1989), while neuronal AChRs formed by α3β2 and α4β2 subunit combinations are sensitive to κ-BGT (Deneris et al., 1988). The sensitivity of the α3β2 AChR to κ-BGT is tenfold greater than the α4β2 complex (Luetje et al., 1990a, b). Interestingly, the α3β4 complex appears to be insensitive to κ-BGT (Duvoisin et al., 1989), indicating that the β-subunit also affects the ligand binding characteristics (see Section X). The α2β2 neuronal AChR is insensitive to both α-BGT and κ-BGT (Wada et al., 1988).

The chicken α7 subunit can form functional homomeric AChRs sensitive to α-BGT (Couturier et al., 1990b; Bertrand et al., 1992). Neuronal α-subunits from *Drosophila* (Sawruck et al., 1990a, b) and locust (Marshall et al., 1990) also form functional homomeric AChRs. It remains to be determined whether other subunits contribute to physiologically relevant AChR complexes comprising α-subunits able to form functional homomeric AChRs. The pharmacology of a homomeric locust AChR (Marshall et al., 1990) is worth noting, as it exemplifies the heterologous ligand-binding properties of the AChRs from species representing different levels of evolution. Locust AChRs composed of only αL1 subunits are blocked by α-BGT, κ-BGT, bicuculline (a $GABA_A$ receptor ligand), and strychnine (a glycine receptor antagonist).

Other neurotoxins can differentially block AChRs. Neosurugarotoxin (NeSuTx), isolated from the Japanese ivory shell, *Babylonia japonica*, blocks AChRs formed by the β2 subunit in combination with the α2, α3, or α4 subunit, whereas

the $\alpha1\beta1\gamma\delta$ AChR is relatively insensitive (Luetje et al., 1990a, b). In contrast, α-conotoxins (α-CnTx) isolated from the venom of marine snails (Gray et al., 1988) block only the $\alpha1\beta1\gamma\delta$ AChR. Lophotoxin, a cyclic diterpene from gorgonian corals (Culver et al., 1985), covalently labels Tyr_{190} of the *Torpedo* α-subunit (Abramson et al., 1989) — a residue conserved in all AChR α-subunits with the notable exception of the neuronal α5 subunit (Boulter et al., 1990a). As predicted from the presence of this tyrosine, AChRs formed by combinations of $\alpha1\beta1\gamma\delta$, $\alpha2\beta2$, $\alpha3\beta3$, and $\alpha4\beta2$ subunits are sensitive to lophotoxin, although the $\alpha2\beta2$ AChR is less sensitive for reasons that remain unclear (Luetje et al., 1990a). A functional AChR has not been expressed successfully in *Xenopus* oocytes using the α5 subunit, which is likely to form a lophotoxin-insensitive complex, given the absence in the α5 subunit sequence of a Tyr residue at position 190 (Boulter et al., 1990a). Table 2 summarizes the toxin sensitivity of AChRs resulting from combinations of different neuronal subunits.

The β-subunit affects the time course and extent of desensitization in a species-specific manner (Sumikawa and Miledi, 1989). Expression of neuronal AChR subunits in *Xenopus* oocytes indicated that both the α- and β-subunits influence agonist sensitivity and desensitization (Cachelin and Jaggi, 1991; Gross et al., 1991; Luetje and Patrick, 1991; Papke and Heinemann, 1991). Coexpression of the chick subunit pairs $\alpha3/n\alpha1$ and $\alpha4/n\alpha1$ demonstrated that the α3 subunit lowers the sensitivity for ACh and enhances desensitization (Gross et al., 1991). Hybrid subunits containing the N-terminal sequence of either the α3 or α4 subunit indicated that the N-terminal region of the α-subunit influences ACh sensitivity (Gross et al., 1991). The β-subunit affects both agonist sensitivity and AChR activation kinetics. The β2 subunit confers sensitivity to cytisine of the AChR formed with the

Table 2
Contribution of Different Subunits to the Toxin Sensitivity of Neuronal AChRs

AChR	Neurotoxin Sensitivity	Ref.
$\alpha1\beta1\gamma\delta$ $\alpha1\beta2\gamma\delta$ α7	α-BGT sensitive	Deneris et al., 1988; Couturier et al., 1990b; Bertrand et al., 1992
$\alpha2\beta2$ $\alpha3\beta4$	α-BGT insensitive κ-BGT insensitive	Deneris et al., 1988; Wada et al., 1988; Duvoisin et al., 1989
$\alpha3\beta2$ $\alpha4\beta2$	α-BGT low sensitivity, κ-BGT sensitive	Deneris et al., 1988; Luetje et al., 1990a, b
$\alpha2\beta2$ $\alpha3\beta2$ $\alpha4\beta2$	NeSuTx sensitive, α-CnTx insensitive	Luetje et al., 1990a, b
$\alpha1\beta1\gamma\delta$	NeSuTx insensitive, α-CnTx sensitive	Luetje et al., 1990a, b
$\alpha1\beta1\gamma\delta$ $\alpha2\beta2$ $\alpha3\beta3$ $\alpha4\beta2$	Lophotoxin sensitive	Luetje et al., 1990a, b

From McLane, K. E., Dunn, S. J. M., Manfredi, A. A., Conti-Tronconi, B. M., and Raftery, M. A. The nicotinic acetylcholine receptor as a model of a superfamily of ligand gated ion channel proteins. **In:** *Handbook for Protein and Peptide Design*, Carey, P. R., Ed.,

α2, α3, or α4 subunits, and the β4 subunit forms cytisine-insensitive AChRs (Luetje and Patrick, 1991). The β-subunit also confers different relative sensitivities to ACh and DMPP (1,1-dimethyl-4-phenylpiperazinium) (Luetje and Patrick, 1991).

Chick sympathetic neurons contain four classes of AChRs and express at least six AChR subunits (α3, α4, α5, α7, β2, and β4). Listerud et al. (1991) used antisense oligonucleotides to selectively delete individual different subunits and determine their functional contribution to cholinergic function. Antisense oligonucleotides against the α3 subunit decreased the number of channel openings of all four classes of AChRs and resulted in the predominance of a new class of channels composed of the α7 subunit and sensitive to α-BGT. Because untreated neurons are α-BGT insensitive, the α7 subunit might form AChR complexes involving other α-subunit subtypes, with different α-BGT-binding properties than the complexes formed by α7 subunit alone.

Expression of mRNAs for the α2, α3, α4, α5, and β2 subunits has been mapped in rat brain by *in situ* hybridization (Wada et al., 1988, 1989; Boulter et al., 1986; Goldman et al., 1986, 1987; Deneris et al., 1988). The α4 and β2 subunits are the most highly and extensively expressed in mammalian brain (Goldman et al., 1987; Deneris et al., 1988; Wada et al., 1988, 1989). Other neuronal AChR subunits have less diffuse expression and may be related to specific functions, although these remain to be determined (Wada et al., 1988, 1989; Duvoisin et al., 1989; Boulter et al., 1990a). Table 3 lists the most prominent regions of hybridization of the different α- and β-probes.

In summary, functional diversity of neuronal AChRs results from different combinations of subunits. This diversity is reflected in differences in channel conductance and open time, sensitivity to neurotoxins, and binding properties for agonists and antagonists. The regional expression of different neuronal AChR subtypes indicates that these different functional properties may be of physiological importance.

Table 3
In Situ Hibridization of AChR Subunit Probes in the Nervous System[a]

CNS region	Probes
Substantia nigra	α3, α4, α5, β2, and β3
Medial habenula	α3, α4, β3, and β4
thalamus	α3, α4, α5, and β2
Trigeminal ganglia	α3, α5, β2, and β3
Interpeduncolar nucleus	α2, α3, and β2
Hippocampus	α2, α3, α4, α5, and β2
Cerebral cortex	α3, α4, α5, and β2
Hypothalamus	α3, α4, and β2
Adrenal medulla	α3, α4, α5, α7, β2, and β4

[b] From Boulter et al., 1986; Deneris et al., 1987, 1988; Duvoisin et al., 1989; Goldman et al., 1986, 1987; Wada et al., 1988, 1989.

From McLane, K. E., Dunn, S. J. M., Manfredi, A. A., Conti-Tronconi, B. M., and Raftery, M. A. The nicotinic acetylcholine receptor as a model of a superfamily of ligand gated ion channel proteins. **In:** *Handbook for Protein and Peptide Design*, Carey, P. R., Ed., Copyright © 1995 by Academic Press, Inc. With permission.

IV. TRANSMEMBRANE TOPOLOGY OF THE AChR SUBUNITS

All *Torpedo* AChR subunits form both extracellular and cytoplasmic domains (Strader, 1979). Because of their sequence similarity, all AChR subunits should have similar transmembrane folding. Hydropathy analysis of a "typical" AChR subunit (reviewed in Claudio, 1989; Stroud et al., 1990) identified a long N-terminal region of ~200 amino acids rich in hydrophilic residues that could form an extracellular domain, followed by four hydrophobic potential α-helices ~20 amino acid long (Figure 1), referred to as M1 to M4, which could cross the membrane (Finer-Moore and Stroud, 1984; Guy, 1983). Between M3 and M4 there is a long sequence region, most diverged in the different AChR subunits, containing a segment,

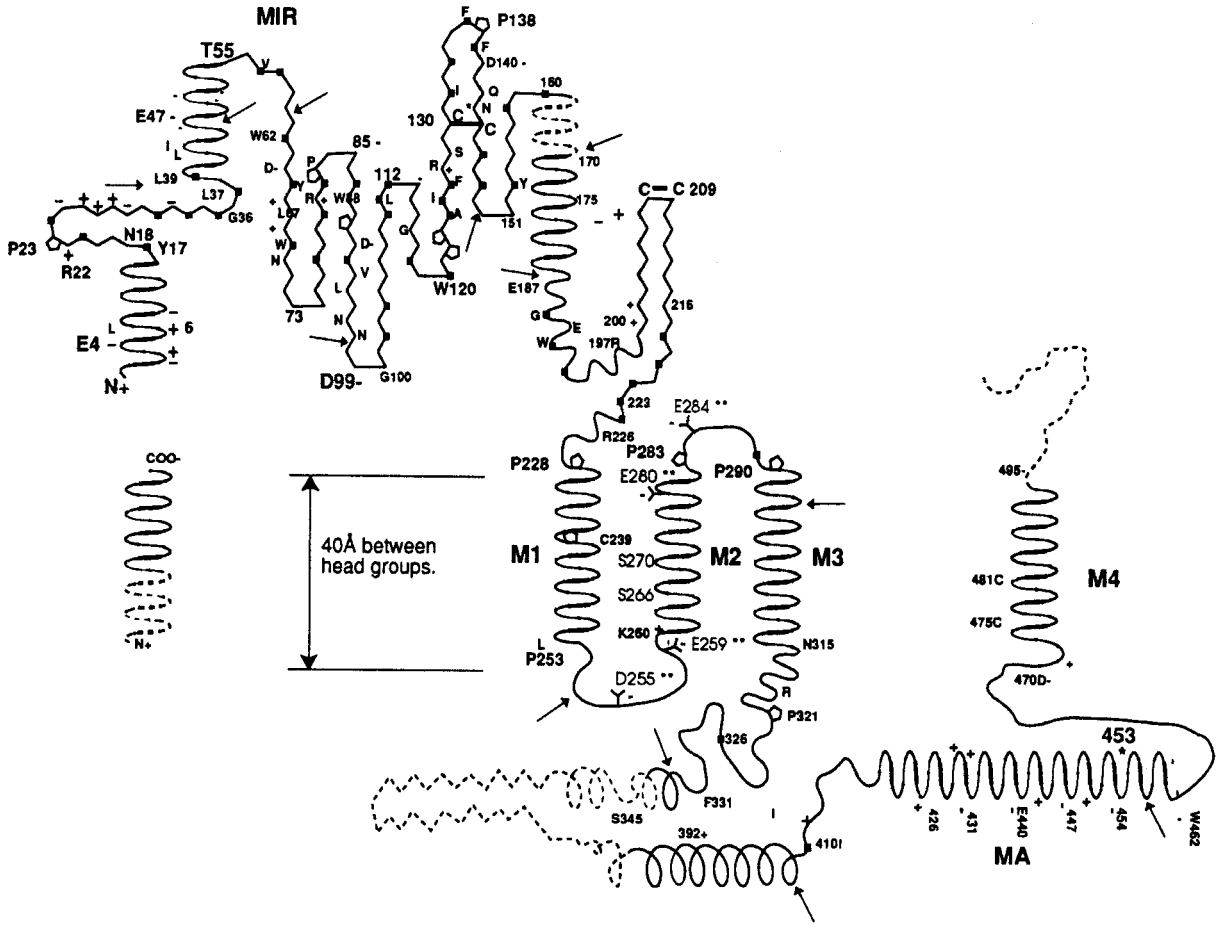

FIGURE 1. Consensus model of a possible topology of a peripheral AChR subunit and secondary structure predicted by amphipatic analysis. The four hydrophobic, putative membrane-spanning α helices are labeled M1 to M4. Letters identify the usual amino acid residue of highly conserved positions. Square blocks identify conservation of hydrophobic residues in the aminoterminal putative extracellular domain that would tend to fold toward the inside. Residues in M2 whose mutation causes a charge in conductance are indicated in narrow letters, with (**) on those that are charged. Positive and negative signs identify regions that generally carry charged side chains. The conserved cysteines, known to form a disulfide-linked loop in all four neuromuscular AChR subunits (indicated as C130-C144, according to the numbering of the consensus alignment, corresponding to Cys_{128} and Cys_{142} of the *Torpedo* α subunit), enclose the conserved site of the N-linked glycosylation site found in all neuromuscular AChR subunits. The adjacent, disulfide-linked cysteines at the agonist binding site, found only in AChR α subunits, are labeled C208 and 209, following the numbering of the consensus alignment (corresponding to Cys_{192} and Cys_{193} of the *Torpedo* α subunit). The MIR is located on the N-terminal putative extracellular domain. Dashed lines indicate sequence regions non-conserved between species, and arrows indicate common intron boundaries. (From Stroud, R. M., McCarthy, M. P., and Schuster, M. 1990. *Biochemistry* **29**, 11009–11023. With permission.)

called MA, which has the periodicity of an amphipatic α-helix (Finer-Moore and Stroud, 1984). M4 is followed by a short carboxyl terminal region.

Different models of the transmembrane folding of the AChR subunits have been proposed, with four (M1 to M4) or five (M1 to M4 and MA) transmembrane segments. In both models, the sequence region preceding M1 is extracellular, while the COOH terminus is extracellular in the four-transmembrane-domain model and cytoplasmic in the five-transmembrane-domain model. We summarize here the experimental evidence supporting either model. For more detailed reviews on these matters see Maelicke, 1988; Claudio, 1989; Stroud et al., 1990; Betz, 1990a, b; Galzi et al., 1991.

A. Region between the Amino Terminus and M1

The *Torpedo* AChR δ-subunit contains a processed signal peptide, indicating that the mature amino terminus is extracellular (Anderson et al., 1982). The amino termini of AChR subunits expressed *in vitro* are translocated into the lumen of microsomal vesicles (topologically equivalent to the extracellular space), as are the amino termini of native AChR subunits (Anderson et al., 1983; Chavez and Hall, 1991).

At least part of the sequence region between the amino terminus and the putative transmembrane segment M1 is extracellular, because it contains (1) an *N*-glycosylation site(s) (Asn_{141} of the muscle-like AChR subunits, Asn_{30}, and sometimes Asn_{141} of neuronal AChR subunits; reviewed in Claudio, 1989), (2) residues involved in the formation of a cholinergic ligand-binding site (see Section III), and (3) a sequence region (within residues 67–76; see Section X.C) that contains important constituent element of the main immunogenic region (MIR) (Bellone et al., 1989) — an extracellular area of the AChR that dominates the autoantibody response in MG (reviewed in Lindstrom et al., 1988).

The transmembrane topology of the N terminus of the α-subunit was investigated by expressing fragments of the α-subunit sequence containing novel glycosylation sites. A fragment terminating at position α_{207} (just before the M1 segment) was a nonintegral membrane protein, and glycosylation sites introduced at position α_{154} and α_{200} were found on the lumenal side of microsomal vesicles (equivalent topologically to the extracellular space). This suggests that the entire N-terminal domain preceding M1 is extracellular (Chavez and Hall, 1991), a conclusion supported by studies on the tridimensional structure of *Torpedo* AChR employing low-dose electron microscopy and X-ray diffraction, which concluded that the volume of protein protruding toward the extracellular space is 215,000 $Å^3$ (157 kDa) — the predicted molecular mass formed by the N-terminal region, up to M1, of all five subunits (Noda et al., 1983c; Finer-Moore and Stroud, 1984) plus the oligosaccharide moieties (Poulter et al., 1989).

B. Potential Transmembrane Segments (M1 to M4 and MA)

The hydrophobic domain M2 is rich in uncharged hydrophilic residues and may contribute to the lining of the ion channel (reviewed in Miller, 1989; Dani, 1989) because, as described in more detail below, mutations of this segment alter the ion conductance properties of AChRs expressed in *Xenopus* oocytes (Imoto et al., 1988; Leonard et al., 1988) and channel blockers label residues within this segment (Hucho, 1986; Hucho et al., 1986; Giraudat et al., 1986, 1989). Synthetic peptides corresponding to the M2 sequence form cation channels in lipid bilayers (Oiki et al., 1988). The M1 segment may also be involved in formation of the channel, as suggested from labeling experiments with noncompetitive blockers (Karlin et al., 1986). A highly conserved proline residue in the middle of the M1 segment of all AChR subunits might be important in conferring structural flexibility and facilitating ion channel gating (Dani, 1989). A transmembrane disposition of M4 is suggested by the selective labeling of Lys residues of AChR in sealed vesicles, in the presence and absence of saponin (Dwyer, 1991); $Lys_{\alpha 380}$, amino terminal to M4, has a cytoplasmic location, while $Lys_{\gamma 486}$, which is carboxyl-terminal to M4, is extracellular.

The potential amphipathic α-helical MA segment (Finer-Moore and Stroud, 1984; Guy, 1983; Stroud et al., 1990) was proposed to contribute to

the lining of the ion channel. A synthetic peptide corresponding to the MA sequence of the *Torpedo* AChR β-subunit forms ion channels in artificial phospholipid bilayers (Ghosh and Stroud, 1991). However, this segment can be deleted from the *Torpedo* sequence without affecting the formation of the ion channel by subunits expressed in *Xenopus* oocytes (Mishina et al., 1985), and antibodies to epitopes within the MA sequence region bind to the cytoplasmic surface of the AChR (Ratnam et al., 1986b; Maelicke et al., 1989). Nonconservative substitution of cysteine residues in the region MA of the α-subunit (residues Cys_{416} and Cys_{420}) with bulky substituents such as phenylalanine had little effect on the function of the resulting mutated AChR, suggesting that the putative helix MA was not functionally involved in ion channel formation (Pradier et al., 1989). That MA may not have a transmembrane disposition is also supported by the disappearance of antibody epitopes comprised of this region after trypsin treatment of native AChR (Roth et al., 1987), and by the results of experiments where the transmembrane disposition of the different putative transmembrane regions of the AChR, including MA, was deduced using proteolysis protection assays of fusion proteins containing a reporter group fused after the nucleic acid sequence encoding each putative transmembrane domain (Chavez and Hall, 1992).

1. Segments M1 and M2 May Be Involved in Formation of the Ion Channel

Hydropathy analysis of the primary structures of *Torpedo* AChR subunits led to suggestions regarding the possible participation of putative transmembrane segments M1 and M2 (Noda et al., 1983c) or M3 (Devillers-Thiery et al., 1983) in the formation of the ion channel. Based on the presence of polar, uncharged amino acids in these putative α-helical domains, it was proposed that M1 and M2 were the most likely candidates for channel formation (Noda et al., 1983c). An alternative model postulated M3 as a channel-forming structure (Devillers-Thiery et al., 1983).

Two main experimental approaches have been used in attempts to correlate the transmembrane segments with channel structure. The first is based on the molecular biological techniques of deletion mutations, point mutations, and the synthesis or chimeric receptors (reviewed in Claudio, 1989). The second involves photoaffinity labeling utilizing channel-specific blockers (reviewed in Karlin et al., 1986; Claudio, 1989; Stroud et al., 1990; Galzi et al., 1991).

The possible role of the four putative transmembrane domains in channel formation was first studied by making a series of deletion mutations in the α-subunit (Mishina et al., 1985). Because deletion of any of these domains yielded nonfunctional AChRs, those experiments did not identify possible channel lining sequences. Chimeric *Torpedo*-calf AChRs were used to investigate the effects of mutations in the δ-subunits (Imoto et al., 1986). Mutations in the M2 region and the adjacent segment linking M2 and M3 profoundly affected channel conductance and conferred *Torpedo*-like or bovine-like channel properties to the resulting chimeric or mutated AChRs (Imoto et al., 1986; see also Hucho, 1986). Removal of a negative charge at the amino terminal (putative intracellular) end of M2 resulted in a reduction of the outward current through the channel and had a lesser effect on the inward current. Conversely, removal of one or more negative charges at the carboxyl-terminal, putative extracellular end of M2 caused a greater reduction of the inward than the outward current (Imoto et al., 1988). Thus, rings of negative charge at either end of this segment are important determinants of channel conductance.

Point mutations were introduced in the M2 segment of mouse AChR to investigate the role of serine residues within this domain on the duration of the block by QX-222, a relatively pure open-channel blocker (Leonard et al., 1988). The apparent affinity of QX-222 decreased proportionally to the number of mutated serines, further implicating M2 in channel formation. These results led to a model in which QX-222 binds within an ion channel formed by M2 helices contributed by each of the subunits (Leonard et al., 1988; Charnet et al., 1990).

Imoto et al. (1991) altered the size and polarity of uncharged polar amino acids between the putative cytoplasmic and extracellular negatively charged rings of M2. They concluded that the

threonine and serine residues contained in the M2 segments of α-, β-, γ-, and δ-subunits line a short, narrow channel close to the cytoplasmic side of the membrane. Further studies on *Torpedo* AChRs expressed in *Xenopus* oocytes, carrying mutations of charged and polar amino acids in three anionic rings (extracellular, intermediate, and cytoplasmic), suggested that amino acids in the intermediate ring may be part of a cation selectivity filter (Konno et al., 1991).

Mutation of amino acid residues to cysteine followed by expression and chemical modification of the new thiol groups has been utilized in structural and functional studies of a number of proteins such as an aspartate-binding protein (Falke et al., 1988; Pakula and Simon, 1992), colicin (Todd et al., 1989; Jakes et al., 1990), and bacteriorhodopsin (Altenbach et al., 1990). This approach has been applied to the M2 region of the mouse muscle α-subunit (Akabas et al., 1992), where nine consecutive residues (at position 241-250) were singly mutated to cysteine, and the resulting mutated α-subunit was expressed in oocytes in combination with wild-type β-, γ-, and δ-subunits. Following chemical modification, voltage-clamp currents were recorded. The results showed that the alternating residues Ser_{248}, Leu_{250}, Ser_{252}, and Thr_{254} are exposed in the closed channel. This suggests a β-strand structure for this region of M2 rather than an α-helix as was generally assumed. Thus, a channel comprised (partly or entirely) of M2 segments from five subunits would consist of some form of β-barrel structure. Given these results, it is a noteworthy caution that predictive methods for the structure of putative membrane spanning regions may err.

The M2 domain has also been implicated in channel formation in the neuronal AChR formed by α7 subunits. Mutation of Leu_{247} to threonine altered activation and desensitization properties and the pharmacological profile of the resulting AChR (Revah et al., 1991; Bertrand et al., 1992). It was suggested that in the desensitized state of wild-type AChR, Leu_{247} blocks the ion channel, but that mutation to a threonine renders this state conductive.

Photoaffinity-labeling studies using noncompetitive inhibitors have been conducted in several laboratories. The local anesthetic, ^{3}H-TPMP$^+$, labels Ser_{262} of the δ-subunit, Ser_{254} of the β-subunit, and Ser_{248} of the α-subunit, all of which are within the M2 domain (Oberthür and Hucho, 1988; Hucho, 1986). ^{3}H-Chlorpromazine has labeled serine residues within the M2 domains of all subunits (positions δ-262, β-254, α-248, and γ-257), in addition to leucines at positions β-257 and γ-260 and threonine at γ-253 (Giraudat et al., 1986, 1987, 1989; Revah et al., 1990). These results agree with those of mutagenesis studies that implicate M2 as a channel-forming sequence. However, as pointed out by DiPaola et al. (1990), there are pitfalls in the interpretation of the photoaffinity-labeling results, as neither TPMP$^+$ nor chlorpromazine have been conclusively demonstrated to bind within the ion channel. Furthermore, the studies summarized above used AChR in the resting or desensitized states rather than the open-channel state.

Time-resolved photoaffinity labeling by ^{3}H-quinacrine azide has been used in an attempt to label AChR in the open-channel conformation (DiPaola et al., 1990). The segment α208-243 was labeled, which encompasses the M1 domain, but not M2. The time frame of these experiments was sufficiently fast (irradiation within 20 ms of mixing AChR with ACh and ^{3}H-quinacrine) to evade the faster phases of desensitization (reviewed in Ochoa et al., 1989). However, under the conditions used for preparative labeling, the saturating concentrations of ACh used make it likely that the flux response would be extremely fast, rendering it debatable whether the channels would still be open at the time of irradiation.

C. Putative Cytoplasmic Domain between the Transmembrane Segments M3 and M4

This sequence region is highly divergent between different α- and β-subtypes (reviewed in Claudio, 1989), and it is particularly long in the α4 subunit (Goldman et al., 1987). This segment may be involved in the differential functional characteristics that each α- and β-subunit confer to the resulting AChR complexes. The carboxyl-terminal part of this sequence region corresponds to the proposed MA amphipatic helix described in Section IV.B. The amino terminal part forms a

cytoplasmic domain, because several studies using sequence-specific antibodies recognizing different parts of this sequence region in different AChR subunits (sequences α304-322, α330-346, α332-350, α339-378, α349-364, α360-378, β350-358, β368-406, and γ360-377), in both *Torpedo* and muscle AChRs, consistently found a cytoplasmic location of antibody binding (Ratnam et al., 1986a, b; Young et al., 1985; Kordossi and Tzartos, 1987; LaRochelle et al., 1985; Lei et al., 1992).

A cytoplasmic disposition of this sequence region is also supported by results of experiments where the transmembrane topology of the different putative transmembrane regions of the AChR, M1-M4 and MA, was deduced using proteolysis protection assays of fusion proteins containing a reporter group fused after the nucleic acid sequence encoding each putative transmembrane domain (Chavez and Hall, 1992).

In conflict with the conclusion that most or all of the region between M3 and M4 is involved in the formation of the cytoplasmic domain, a study that determined the sequence of AChR fragments released after brief proteolytic treatment of sealed AChR-rich membrane vesicles concluded that the sequences α341-380, β351-385, γ353-414, and δ328-341, fragments of which were quickly released by trypsin treatment, are exposed on the extracellular surface (Moore et al., 1989).

D. Carboxyl Terminus

The transmembrane disposition of the COOH terminus of the AChR subunits has been investigated by immunological, biochemical, and genetic approaches.

The results of studies using monoclonal antibodies directed against the carboxyl terminus of different AChR subunits consistently supported a cytoplasmic location of the carboxyl terminus, because the antibodies bound to the cytoplasmic side of the postsynaptic membrane (Young et al., 1985), or, in immunochemical assays, bound only after treatment of the AChR-rich sealed membrane microsacs with membrane-permeabilizing agents (Ratnam and Lindstrom, 1984; Lindstrom et al., 1984). An alternative explanation of the latter results could be that the carboxyl terminus is extracellular but not available to antibody binding in its native conformation.

Opposite results were consistently obtained by nonimmunological approaches. Several studies took advantage of the fact that *Torpedo* AChRs exist as dimers held together by a disulfide bridge occurring between the penultimate residue of the δ-subunit of each monomer, which are cysteines (DiPaola et al., 1988). Different studies using membrane-impermeable agents and sealed AChR-containing vesicles all indicated that disulfide bridges holding together AChR dimers can be reduced from the extracellular surface (Dunn et al., 1986; McCrea et al., 1987; DiPaola et al., 1988, 1989). In another study (Dwyer, 1991), residue Lys_{486} of the γ-subunit, which is on the carboxyl-terminal side of the transmembrane domain M4, could be labeled with membrane-impermeable reagents in sealed AChR-rich microsacs from *Torpedo* electric organ, indicating that this residue, and therefore the carboxyl terminus of the γ-subunit, is extracellular.

A recent study (Chavez and Hall, 1992) investigated the location of the carboxyl terminus of the α- and δ-subunits of mammalian muscle using fusion proteins containing a prolactin reporter sequence attached downstream of the M4 region. The orientation of the prolactin domain relative to the microsomal membrane was determined by a proteolysis protection assay. This study concluded that the carboxyl termini of the α- and δ-subunits are located on the lumenal side of microsomal vesicles, which is the topological equivalent of the extracellular space.

V. PENTAMERIC QUATERNARY STRUCTURE OF THE AChRs

The muscle-type AChR of *Torpedo* electric organ is composed of four subunits — α, β, γ, and δ. The subunit stochiometry of *Torpedo* AChR was directly determined by two different approaches. Amino acid analysis of subunits extracted from analytical sodium dodecyl sulfate (SDS) gels first indicated that the relative ratio of the α-, β-, γ-, and δ-subunits is approximately 2:1:1:1 (Lindstrom et al., 1979). Simultaneous

quantitative amino-terminal microsequencing of the subunits present in SDS denaturated purified AChR (Raftery et al., 1980), directly demonstrating that the four subunits form a pseudosymmetric pentameric complex $(\alpha)_2\beta\gamma\delta$. A similar subunit stoichiometry was obtained for the AChRs from *Electrophorus* electric organ (Conti-Tronconi et al., 1982a) and from fish, calf, and chicken muscle (Conti-Tronconi et al., 1982b, 1984, and unpublished observations).

Functional neuronal AChRs can be either homomeric complexes, such as the complexes of the chick brain α7 subunit (Couturier et al., 1990b) and the α-BGT-sensitive AChRs of *Drosophila* (Sawruck et al., 1990a, b) and locust (Marshall et al., 1990), or heteromeric complexes composed of α- and β-subunits (reviewed in Deneris et al., 1991). A pentameric structure for the neuronal AChRs is implied by the molecular size of the intact receptors (Conti-Tronconi et al., 1985; Smith et al., 1985; Whiting and Lindstrom, 1986, 1987, 1988; Whiting et al., 1987a, 1991). Direct evidence for a pentameric structure for the α4/β2 AChR subtype has been provided using two different approaches. Quantitative ^{35}S-methionine incorporation was used by Lindstrom and coworkers to determine the stoichiometry of α4 and β2 subunits expressed in fibroblasts (Whiting et al., 1991) and *Xenopus* oocytes (Anand et al., 1991). Their results indicated a relative subunit ratio of 2:3 for the α4 and β2 subunits, respectively. The same subunit stoichiometry was determined using site-directed mutagenesis of the α4 and β2 subunits to create hybrid AChR complexes with distinguishable electrophysiological characteristics (Cooper et al., 1991). By quantitating the hybrid channels formed, a pentameric complex of $(\alpha4)_2(\beta2)_3$ was deduced.

VI. TRIDIMENSIONAL STRUCTURE OF THE AChR FROM *TORPEDO* ELECTRIC ORGAN

The tridimensional shape of the AChR from *Torpedo* electric organ has been determined by different approaches, including electron microscopy and low-dose electron microscopy of negatively stained AChR molecules — obtaining both top views of the extracellular portion of the AChR and lateral views of individual molecules — metal replicas of freeze-fractured or freeze-etched postsynaptic membranes, X-ray diffraction of two-dimensional crystalline arrays of AChR in postsynaptic membrane fragments, and, more recently, scanning tunneling microscopy (STM) imaging. Detailed reviews of these structural studies are reported in Mitra et al. (1989), Unwin et al. (1988), Stroud, et al. (1990), and Bertazzon et al. (1992). We summarize here only the overall conclusions of those studies, which all yielded consistent, similar pictures of the AChR, although at different resolutions (between 22 and 11.25 Å in the more recent studies [Mitra et al., 1989; Unwin et al., 1988; Bertazzon et al., 1992]), and consequently with different degrees of structural detail.

The AChR is almost cylindrical (mean diameter of ~65 Å in the extracellular and transmembrane part, while it is narrower on the cytoplasmic side [Brisson and Unwin, 1985]). The total length of the AChR was originally estimated to be ~140 Å (Brisson and Unwin, 1985). In improved density maps obtained by low-density electron microscopy and X-ray diffraction (Mitra et al., 1989), the estimated total length of the molecule was 115 Å in native AChR and 130 Å after alkali treatment (the increased dimensions are probably due to disordering of the protein domains induced by alkali treatment).

Native, nondesensitized AChR molecules are almost perfectly symmetrical: all subunits are rod-shaped structures that lie approximately perpendicular to the plane of the membrane. They are arranged symmetrically around the central pore and extend radially toward the surrounding lipids by the same distance from the center (Unwin et al., 1988; Mitra et al., 1989). They surround a water-filled opening, presumed to be the ion channel, along the axis of the AChR molecule (Kistler et al., 1982); Brisson and Unwin, 1985). The pentagonal symmetry of the AChR is most perfect in the transmembrane region and over the contiguous regions of the extracellular side (Brisson and Unwin, 1985; Unwin et al., 1988). Exposure to carbamylcholine (Carb) and consequent AChR desensitization reduces the AChR symmetry because of rearrangement and protru-

sion from the center of two subunits that were proposed to be the γ- and δ-subunits, assuming that the β-subunit lies between the two α-subunits (Unwin et al., 1988), and assumption that may or may not be true (Claudio, 1989). The almost perfect pentameric symmetry of the AChR is also indicated by the angle separating the two α-subunits (144 ± 4°), which corresponds precisely to two sectors of a pentagon (Fairclough et al., 1983). Such symmetry and the strong sequence homology of the AChR suggest that the individual AChR subunits fold across the membrane in a similar way and that they have similar tertiary structure along the subunit/subunit interfaces. The symmetric organization might have an important role for coordinated movement of all subunits around the channel and switching to different functional states in response to ligand binding (Brisson and Unwin, 1985), as it occurs to the gap junction subunits after exposure to physiological stimuli (Unwin and Ennis, 1984). The lateral dimensions of the AChR in its transmembrane portion (cross-sections in this region have an area of 540 to 550 Å^2) is compatible with the close packing of four or five α-helices extending through the membrane (Brisson and Unwin, 1985; Stroud et al., 1990).

The AChR subunits are asymmetrically placed in relation to the bilayer because they have two to three times more of their combined mass on the synaptic than on the cytoplasmic side (Kistler et al., 1982; Brisson and Unwin, 1985). A relatively detailed tridimensional structure of the extracellular domain of the AChR has been obtained to date by electron microscopy and STM approaches. It forms a cylindrical "vestibule", which extends 54 Å above the plane of the membrane (Mitra et al., 1989) in the native postsynaptic membrane. The individual height of the subunits above the plane of the synaptic membrane ranges from 50 to 60 Å, and the total outer diameter from 74 to 81 Å. The part of the channel contained in this vestibule is relatively uniform in diameter (25.5 Å in Mitra et al. [1989] and 30 Å in Brisson and Unwin [1985]), while it becomes more narrow and difficult to follow as it approaches the cytoplasmic domain (Kristel et al., 1982; Brisson and Unwin, 1985). The walls surrounding the cylindrical channel contained in the vestibule are 24.5 ± 1.5 Å thick (Stroud et al., 1990), which would exactly accommodate the dimensions of an antiparallel β-barrel structure predicted on the basis of amphipatic secondary structure analysis for the extracellular domain of *Torpedo* AChR (Finer-Moore and Stroud, 1984).

The structure summarized above emerging from electron imaging and X-ray diffraction of AChR-rich membranes has been recently confirmed and detailed by STM imaging of *Torpedo* postsynaptic membrane fragments (Bertazzon et al., 1992), which yielded a detailed picture of the extracellular domain of the AChR. The average outer diameter is 69 ± 10 Å, while the central cavity, taken on contour maps of filtered images at the largest delimiting line, is 26 ± 7 Å. Contour maps of STM images of single molecules yielded further structural details. The total height above the background is 50 Å, close to the dimensions reported earlier for the extracellular moiety of the molecule (Brisson and Unwin, 1985; Mitra et al., 1989; Stroud et al., 1990). Five peaks can be observed, two of which are poorly resolved. The major peak protrudes about 15 Å from the largest contour line delimiting the central pit. On the opposite side of the central opening, a second peak protrudes ~9 Å. On the left side of the AChR molecule (as defined by the two above peaks), there are two poorly resolved peaks: the lower protrudes 3 Å and the upper protrudes 9 Å above this same plane. On the right side, a peak protruding to ~6 Å can be observed. It may be assumed that these five peaks are related to the five subunit domains. The average width of the walls of the pseudosymmetric rosette surrounding the central pit is 25 Å. This contour image closely matches those previously reported from hybrid maps (Mitra et al., 1989).

VII. STRUCTURE OF CHOLINERGIC LIGAND-BINDING SITES: STUDIES WITH AFFINITY LABELS AND EXPRESSION OF MUTANT AChR SUBUNITS

The AChR has two distinct binding sites for agonists and competitive antagonists that are

believed to reside primarily on the two α-subunits. The α-subunit of *Torpedo* AChR was identified as containing an α-BGT binding site by ^{125}I-α-BGT labeling of protein blots after SDS gel electrophoresis (Haggerty and Froehner, 1981; Tzartos and Changeux, 1983; Gershoni et al., 1983). Also, *Torpedo* AChR expressed in *Xenopus* oocytes does not bind α-BGT unless the α-subunit is expressed (Mishina et al., 1984). Other AChR subunits may contribute to the formation of these and perhaps other cholinergic sites, as discussed in Section X.

A. Potential Sulfhydryl/Disulfide Groups Involved in Ligand Binding

A sulfhydryl group within 1 nm of the binding site for ACh on the AChR is specifically labeled with cholinergic affinity labels such as 4-(*N*-maleimido)benzyltri[^{3}H]-methylammonium (^{3}H-MBTA) and bromoacetylcholine (BAC) (reviewed in Maelicke, 1988; Claudio, 1989). Alkylation with these ligands blocks α-BGT binding and AChR function. They label the AChR α-subunit (reviewed in Maelicke, 1988; Claudio, 1989).

Three potential disulfide bonds exist in the *Torpedo* AChR α-subunit that can be labeled with disulfide reagents and distinguished on the basis of their sensitivity to reduction: they are formed by the Cys pairs Cys_{128}/Cys_{142}, Cys_{412}/Cys_{418}, and Cys_{192}/Cys_{193} (Kao et al., 1984; Kao and Karlin, 1986; Mosckovitz and Gershoni, 1988). Cys_{128}/Cys_{142} and Cys_{192}/Cys_{193} are within the amino-terminal putative extracellular domain, and Cys_{412}/Cys_{418} within M4 (Noda et al., 1982). A free cysteine at position 222, which when alkylated blocks the ion channel, has been associated with a hydrophobic pocket (Huganir and Racker, 1982; Yee et al., 1986; Clarke and Martinez-Carrion, 1986). Models have been proposed in which Cys_{128}/Cys_{142} or Cys_{192}/Cys_{193} were within the cholinergic binding site (Noda et al., 1982; Smart et al., 1984; Criado et al., 1985; Kao et al., 1984; Kao and Karlin, 1986).

That Cys_{128}/Cys_{142} could be within the cholinergic site was first proposed based on theoretical modeling of the AChR sequence (Noda et al., 1982; Smart et al., 1984). Mutations of either Cys_{128} or Cys_{142} of the *Torpedo* α-subunit resulted in undetectable levels of α-BGT binding and no detectable response to ACh (Mishina et al., 1985). However, Sumikawa and Gehle (1992) demonstrated that mutation of either Cys_{128} or Cys_{142} causes a lower efficiency of complex assembly and membrane incorporation, but the few complexes that do form with either mutant α-subunit are responsive to ACh, although insensitive to α-BGT. Therefore, in the native AChR, an intact disulfide bond of the Cys loop is important for formation or accessibility of the α-BGT binding site.

Residues Cys_{192} and Cys_{193} of the *Torpedo* α-subunit were identified by amino acid sequencing as the disulfide pair specifically labeled by the cholinergic affinity labels ^{3}H-MBTA and ^{3}H-*p*-(dimethylamino)-benzenediazonium fluoroborate (DDF) following reduction (Kao and Karlin, 1986; Dennis et al., 1988). The ability of α-BGT and MBTA to mutually inhibit labeling (Kao et al., 1984; Gershoni et al., 1983) suggests that they bind to a common site. When either Cys_{192} or Cys_{193} were mutated to serine and coexpressed with the other *Torpedo* AChR subunits in *Xenopus* oocytes, α-BGT binding was reduced by 60 to 70% (Mishina et al., 1985). However, the role of a disulfide bond between Cys_{192} and Cys_{193} in the formation of this ligand-binding site remains elusive. In the intact *Torpedo* AChR, reduction, or reduction and alkylation, does not change the number of α-BGT binding sites (Moore and Raftery, 1979; Walker et al., 1981). Similarly, selective alkylation of the vicinal cysteine residues with a large adduct does not interfere with the binding of α-BGT to the intact *Torpedo* α-subunit (Mosckovitz and Gershoni, 1988). Expression studies in *Xenopus* oocytes of *Torpedo* α-subunits carrying mutations of Cys_{192} or Cys_{193} also indicated that α-BGT binding can occur in the absence of an intact disulfide (Mishina et al., 1985). In addition, the consensus of the results from binding studies of α-BGT to synthetic peptides (discussed in Section VIII.A) indicates that, although the sequence segment surrounding the vicinal Cys_{192} and Cys_{193} has an important role in forming a

binding site for α-BGT, a vicinal disulfide bridge between Cys_{192} and Cys_{193} is not critical for α-BGT binding.

B. Other Amino Acid Residues in Proximity to Cholinergic Ligand-Binding Sites Identified by Affinity Labeling

Studies employing affinity labeling identified other amino residues within the amino-terminal extracellular domain of the *Torpedo* α-subunit as involved in the formation of cholinergic binding sites. Lophotoxin competes directly with the α-BGT binding and covalently labels Tyr_{190} of the AChR α-subunit via a reactive epoxide linkage (Abramson et al., 1989). The photoaffinity cholinergic reagent DDF labels Tyr_{190} as efficiently as Cys_{192} and Cys_{193}, and to a lesser extent Trp_{149} and Tyr_{93} (Dennis et al., 1988; Galzi et al., 1990).

Amino acid residues involved in the formation of an agonist binding site(s) have been identified by affinity labeling of *Torpedo* AChR with ^{3}H-ACh mustard (Middleton and Cohen, 1991) and a ^{3}H-nicotine photoaffinity analog (Cohen et al., 1991). ^{3}H-ACh (Middleton and Cohen, 1991) and ^{3}H-nicotine (Cohen et al., 1991) specifically labeled Tyr_{93} and Tyr_{198} of the α-subunit, respectively. Therefore, a binding site for ACh may be distinct from that for nicotine and other agonists, and formation of these (sub)sites may involve several sequence regions of the α-subunit. This conclusion is supported by studies using monoclonal antibodies recognizing different epitopes within the cholinergic binding site. Competition studies using such antibodies indicate that different subregions within the cholinergic site may form the ligand binding interfaces for Carb, α-BGT, and δ-tubocurare (Watters and Maelicke, 1983; Mihovilovic and Richman, 1987). For example, antibodies that blocked both α-BGT and Carb binding to *Torpedo* AChR did not affect δ-tubocurare binding, thus distinguishing between agonist and antagonist subsites, while another antibody did not affect agonist binding but inhibited ~50% of α-BGT binding and the high-affinity δ-tubocurare binding.

VIII. STRUCTURE OF CHOLINERGIC LIGAND-BINDING SITES: IDENTIFICATION OF SEQUENCE SEGMENTS AND INDIVIDUAL RESIDUES CONTRIBUTING TO CHOLINERGIC BINDING SITES ON THE α-SUBUNIT BY THE USE OF PROTEOLYTIC, SYNTHETIC, AND BIOSYNTHETIC PEPTIDES

A. Studies on *Torpedo* AChR and Muscle AChRs from Different Species

1. *The* Torpedo *AChR* α-*Subunit*

Several laboratories have used proteolytic fragments (Wilson et al., 1984, 1985; Pederson et al., 1986; Oblas et al., 1986; Neumann et al., 1986a), synthetic peptides (Neumann et al., 1986b; Ralston et al., 1987; Wilson et al., 1988; Wilson and Lentz, 1988; Conti-Tronconi et al., 1988, 1989, 1990a, 1991; McLane et al., 1990a, b, 1991a–d, 1993; Gotti et al., 1987, 1988; Greisman et al., 1990), and biosynthetic peptides (Barkas et al., 1987; Aronheim et al., 1988; Ohana and Gershoni, 1990; Ohana et al., 1991) to locate sequence regions of the *Torpedo* AChR α-subunit able to bind α-BGT and perhaps other cholinergic ligands. Such continuous peptide sequences, able to form independent ligand-binding sites in the absence of surrounding structural elements, have been called "prototypes" (Wilson et al., 1988).

Proteolytic mapping of the *Torpedo* AChR α-subunit was initially used to study the cholinergic and α-BGT binding site(s) (Wilson et al., 1984, 1985; Oblas et al., 1986; Pederson et al., 1986; Neumann et al., 1985, 1986a). The results of those studies are summarized in Table 4.

Synthetic peptides have been used widely to investigate the sequence requirements of the cholinergic site that binds α-BGT. Atassi and coworkers identified several sequence segments of the *Torpedo* AChR α-subunit that bound α-BGT — α125-148, α182-198, and α388-408 (McCormick and Atassi, 1984; Mulac-Jericevic and Atassi, 1987; Atassi et al., 1988). While α-BGT binding to peptides containing the Cys-Cys loop (Cys_{128}/Cys_{142}) and the sequence

Table 4
Proteolytic Mapping of the Cholinergic Binding Site on the *Torpedo* α Subunit Using V8 Protease

Fragment binding α-BGT	Residues identified within the peptide fragment	Other binding ligands	Ref.
18 kDa	Asn_{141}, Cys_{128}, Cys_{142}	None	Pederson et al., 1986
20 kDa	Cys_{192}, Cys_{193}	d-TC	Pederson et al., 1986
17 kDa	Asn_{141}, Cys_{128}, Cys_{142}	None	Oblas et al., 1986
19 kDa	Cys_{192}, Cys_{193}	MBTA	Oblas et al., 1986
18 kDa	Cys_{192}, Cys_{193}	MBTA	Wilson et al., 1984, 1985
18 kDa	Residues 169-181	None	Neumann et al., 1985, 1986a

From McLane, K. E., Dunn, S. J. M., Manfredi, A. A., Conti-Tronconi, B. M., and Raftery, M. A. The nicotinic acetylcholine receptor as a model of a superfamily of ligand gated ion channel proteins. **In:** *Handbook for Protein and Peptide Design*, Carey, P. R., Ed.,

α388-408 has not been reproducible, several studies confirmed that the sequence region of the *Torpedo* α-subunit flanking the vicinal Cys residues at position 192 and 193 forms a prototype for α-BGT (Neumann et al., 1986b; Ralston et al., 1987; Wilson et al., 1988; Wilson and Lentz, 1988; Conti-Tronconi et al., 1988, 1989, 1990a, 1991; McLane et al., 1991a; Gotti et al., 1987, 1988; Greisman et al., 1990). The results of those studies are summarized in Table 5.

Conti-Tronconi et al. (1989, 1990a) further defined the structural elements of α-BGT binding site(s) on *Torpedo* AChR by testing a panel of overlapping synthetic peptides corresponding to the complete α-subunit sequence. Two sequence segments corresponding to α55-74 and α181-200 bound ^{125}I-α-BGT and several monoclonal antibodies that compete for α-BGT binding (Watters and Maelicke, 1983; Fels et al., 1986). Therefore, both sequence segments may contribute to a cholinergic binding site recognized by α-BGT: the multipoint attachments of α-BGT to the α-subunit is the structural basis of the high-affinity α-neurotoxin/AChR complex.

The structural characteristics of the *Torpedo* peptides α55-74 and α181-200 have been studied by circular dichroism (CD) and fluorescence spectroscopy (Conti-Tronconi et al., 1991; Raftery et al., unpublished observations). Both peptides have a high content of β-sheet and β-turn (Table 6). Differential CD spectroscopy, in the presence and absence of α-BGT, indicates that peptides α55-74 and α181-200 undergo structural changes upon α-BGT binding, with a net increase in the β-structure component (Conti-Tronconi et al., 1991). These structural changes may reflect a mechanistic basis for the essentially irreversible inactivation of the AChR by α-BGT.

Noncompetitive AChR inhibitors bind to at least three sites on the *Torpedo* AChR: (1) a binding site for ACh, (2) a high-affinity site within the ion channel, and (3) several low-affinity binding sites. Sequence regions contributing to binding sites for the noncompetitive inhibitor phencyclidine (PCP) have been identified using synthetic peptides corresponding to amino acid residues α72-227 on the *Torpedo* AChR α-subunit (Donnelly-Roberts and Lentz, 1991). PCP bound a 56-mer synthetic peptide (α172-227) and two shorter peptide segments (α173-204 and α205-227). Two distinguishable sites that bind PCP within this region were identified — a low-affinity site within amino acid residues α173-204, competitive with α-BGT, and a high-affinity site within residues α205-227. The latter segment contains the transmembrane segment M1, which may be exposed to the inner lining of the ion channel (as discussed in Section IV.B.1). Both high- and low-affinity PCP binding were inhibited by other noncompetitive inhibitors, chloropromazine, tetracaine, and dibucaine, suggesting that PCP specifically binds to a common binding site for noncompetitive inhibitors. This study clearly indicated that the use of synthetic peptide

Table 5
Mapping of the Cholinergic Binding Site on the α Subunit of AChRs from *Torpedo* Electric Organ and Vertebrate Muscle Using Synthetic Peptides and Fusion Proteins

	Source	Apparent K_d or IC_{50}[a]	Type of assay	Ref.
		Synthetic Peptides		
Isolated α subunit[b]	*Torpedo*	100-200 n*M*	Membrane blot assay	Gershoni et al., 1983; Haggerty and Froehner, 1981; Oblas et al., 1986
α173-204	*Torpedo*	500 n*M*; IC_{50} for d-TC: 2 m*M*	Membrane blot assay	Wilson et al., 1985
α185-196	*Torpedo*	35 μ*M*; inhibited by 10 m*M* d-TC	Peptide-conjugated sepharose	Neumann et al., 1986b
α125-148	*Torpedo*, human	150 n*M*	Peptide-conjugated sepharose	McCormick and Atassi, 1984
α182-198	*Torpedo*	IC_{50}: 70 n*M*	Peptide-conjugated sepharose	Mulac-Jericevic and Atassi, 1986
α388-408	*Torpedo*	IC_{50}: 100 n*M*	Peptide-conjugated sepharose	Atassi et al., 1988
α[Lys]388-408	*Torpedo*	1 μ*M*; IC_{50} for d-TC: 500 m*M*	Peptide-conjugated sepharose	Gotti et al., 1987
α172-205	*Torpedo*	200 n*M*	Membrane blot assay	Ralston et al., 1987
α185-199	*Torpedo*	20 μ*M*	Membrane blot assay	Ralston et al., 1987
α173-204	*Torpedo*	500 n*M*	Membrane blot assay	Wilson et al., 1985
AChR	*Torpedo*	0.4 n*M*	Plate assay	Wilson et al., 1988; Wilson and Lentz, 1988
Isolated α subunit	*Torpedo*	46 n*M*	Plate assay	Wilson et al., 1988; Wilson and Lentz, 1988
α173-204	*Torpedo*	42 n*M* (10 n*M* with 0.01% SDS); IC_{50}: 86 μ*M* for d-TC, 8 m*M* for nicotine, 16 m*M* for NaCl 44 n*M* for α-cobratoxin	Plate assay	Wilson et al., 1988; Wilson and Lentz, 1988
α181-198	*Torpedo*	20 μ*M*	Plate assay	Wilson et al., 1988; Wilson and Lentz, 1988
α185-196		24 μ*M*		
α193-204		24 μ*M*		
α173-180		>100 μ*M*		
α194-204		>100 μ*M*		
α179-192		>100 μ*M*		
AChR[c]	*Torpedo*	3 n*M*	Competition assay with *Torpedo* AChR	Wilson et al., 1988
Isolated α subunit	*Torpedo*	10 n*M*	Competition assay with *Torpedo* AChR	Wilson et al., 1988
α173-204	*Torpedo*	100 n*M*	Competition assay with *Torpedo* AChR	Wilson et al., 1988
α181-198	*Torpedo*	IC_{50}: 9 μ*M*	Competition assay with *Torpedo* AChR	Wilson et al., 1988
α179-192		IC_{50}: 17 μ*M*		
α185-196		IC_{50}: 13 μ*M*		
α186-196		IC_{50}: 22 μ*M*		
α193-204	*Torpedo*	87 μ*M*	Competition assay with *Torpedo* AChR	Wilson et al., 1988
α173-180		>400 μ*M*		
α194-204		>500 μ*M*		
α181-200	*Torpedo*	1–3 μ*M*	Membrane blot assay	Conti-Tronconi et al., 1990a, 1991

Table 5 (continued)
Mapping of the Cholinergic Binding Site on the α Subunit of AChRs from *Torpedo* Electric Organ and Vertebrate Muscle Using Synthetic Peptides and Fusion Proteins

	Source	Apparent K_d or IC_{50}[a]	Type of assay	Ref.
		Synthetic Peptides (continued)		
α181-199	Human	25 μ*M*	Membrane blot assay	Griesman et al., 1990
α185-199		80 μ*M*		
α193-208		Inactive		
α177-192		40% binding of α185-199		
α173-204	Human	1 μ*M* (861 n*M* with 0.01% SDS); IC_{50} for d-TC: 48 μ*M*	Plate assay	Wilson and Lentz, 1988
α173-204	Bovine	226 n*M* (48 n*M* with 0.01% SDS); IC_{50} for d-TC: 250 μ*M*	Plate assay	Wilson and Lentz, 1988
α179-191[d]	Human	αCTX 100 n*M* ErabuT α inactive	Quenching of intrinsic Trp fluorescence	Radding et al., 1988
α179-191	Bovine	αCTX 50 n*M* ErabuT α 50 n*M*	Quenching of intrinsic Trp fluorescence	Radding et al., 1988
α181-200	Frog	IC_{50}: 1–2 μ*M*	Competition assay with Torpedo AChR	McLane et al., 1991b
α181-200	Chicken	IC_{50}: 1–2 μ*M*		
α181-200	Mouse	IC_{50}: ~150 μ*M*		
α181-200	Calf	IC_{50}: ~150 μ*M*		
α181-200	Human	IC_{50}: ~150 μ*M*		
α181-200	Cobra	Inactive		
		Fusion Proteins		
α160-216	Mouse	75 n*M*	Filtration assay	Barkas et al., 1987
Isolated α subunit	*Torpedo*	~20 n*M*	Filtration assay	Aronheim et al., 1988
α166-315		~20 n*M*		
α166-200		~20 n*M*		
α184-196		~400 n*M*		
α184-200	*Torpedo*	~20 n*M* IC_{50}: 600 μ*M* DTC IC_{50}: 20 m*M* Carb IC_{50}: 3 μ*M* αCTX	Membrane blot assay	Aronheim et al., 1988
α183-204	*Torpedo*	63 n*M*	Membrane blot assay	Ohana and Gershoni, 1990
	Xenopus	536 n*M*		
	Chick	150 n*M*		
	Mouse	3200 n*M*		
	Calf	6200 n*M*		
	Human	6470 n*M*		
	Cobra	Inactive		Ohana et al., 1991

[a] K_ds were obtained when direct binding of α-BGT (or α-cobratoxin or erabutoxin-α; see footnote b) to peptides immobilized on a solid support was measured. IC_{50}s were obtained when competition assays were used, measuring the ability of the synthetic or biosynthetic sequence to compete with native AChR for cholinergic ligand binding. Unless specified (see footnote #2), α-BGT binding was measured.

[b] The affinity of α-BGT for the isolated α subunit of *Torpedo* AChR, measured in the same type of assay as the peptides listed in the box below, is reported for the sake of comparison.

[c] The affinity of α-BGT for the intact *Torpedo* AChR, measured in the same type of assay as the peptides listed in the box below, is reported for the sake of comparison.

[d] In these studies, the binding of α-cobratoxin and erabutoxin-α, not of α-BGT, was assessed.

From McLane, K. E., Dunn, S. J. M., Manfredi, A. A., Conti-Tronconi, B. M., and Raftery, M.A. The nicotinic acetylcholine receptor as a model of a superfamily of ligand gated ion channel proteins. **In:** *Handbook for Protein and Peptide Design*, Carey, P. R., Ed.,

Table 6
Secondary Structure of Synthetic Sequences of *Torpedo* AChR α Subunit That Bind α-BGT, in the Absence and Presence of Denaturants

Synthetic sequence	α-Helix	β-Sheet	β-Turn	Random Coil
α55-74[a]	0	64.1	10.9	25
α55-74 (GuCl, 7 *M*)	0	20.8	30.9	48.3
α181-200[b]	0	68.1	14.8	17.1
α181-200[c]	11.5	47.7	28.3	12.5
α181-200 (DTT, 10 m*M*)[c]	8.5	58.3	27.1	16.1
α181-200 (GuCl, 7 *M*)	0	76.5	12.3	11.2

[a] 1 mg/ml in 10 m*M* TrisHCl buffer, pH 8.0.
[b] At pH 4.2.
[c] At pH 8.2.

From McLane, K. E., Dunn, S. J. M., Manfredi, A. A., Conti-Tronconi, B. M., and Raftery, M. A. 1993. The nicotinic acetylcholine receptor as a model of a superfamily of ligand gated ion channel protein. **In:** *Handbook for Protein and Peptide Design*, Carey, P. R., Ed., Copyright © 1995 by Academic Press, Inc. With permission.

sequences is a powerful approach for studying the binding sites of multiple ligands.

2. Muscle AChR α-Subunits

^{125}I-α-BGT binds to the sequence regions of human and calf muscle AChR α-subunits flanking Cys_{192}/Cys_{193} (Gotti et al., 1988; Griesman et al., 1990; Wilson and Lentz, 1988). The α-BGT binding properties of synthetic peptides corresponding to the sequence segment 181-200 from muscle AChR α-subunits of different species have been studied (McLane et al., 1991a) (Table 5). The *Torpedo*, frog, and chicken muscle synthetic sequences bound ^{125}I-α-BGT with relatively high affinity (apparent IC_{50}: 1 to 2 μ*M*), whereas the mammalian muscle peptides (human, murine, and bovine) had lower affinity (apparent IC_{50}: ~15 μ*M*) (McLane et al., 1991a). The use of a homologous peptide corresponding to the cobra muscle AChR α-subunit demonstrated that the snake insensitivity to its own toxin may be due to the inability of this sequence segment to form a prototype for α-BGT (McLane et al., 1991a).

A disulfide between Cys_{192} and Cys_{193} exists in the native *Torpedo* α-subunit (Kellaris et al., 1989). A role of such a disulfide in α-BGT binding to the synthetic sequences α181-200 of muscle AChRs from different species was tested by cysteine/cystine modifications (McLane et al., 1991a). Reduction and alkylation reduced α-BGT binding, whereas oxidation to a disulfide-containing peptide monomer had no effect. Therefore, a vicinal disulfide is a possible structure formed by the synthetic peptides but is not critical for α-BGT binding.

Nuclear magnetic resonance (NMR) spectroscopy studies of the low-affinity interaction of ACh with the peptide sequences *Torpedo* α184-200 and human α183-206 expressed in a TrpE fusion protein (Fraenkel et al., 1991) indicated that the quaternary ammonium methyl groups of ACh interact with Trp_{184} of both peptides. This suggests that the α-BGT binding site formed by this sequence region is also a competitive binding site for ACh.

B. Studies on Different Neuronal AChR Subtypes

1. κ-Toxin-Sensitive Neuronal AChRs

The deduced amino acid sequences for a number of α- and β-subunits of avian and rodent

neuronal AChRs have been reported (see Section III.A.2 and Table 1). Different α/β subunit pairs affect the pharmacological profile of the resulting AChR complex (Luetje et al., 1990b; Papke et al., 1989; see also Section III.B and Table 2).

Using overlapping peptides corresponding to the α3 subunit sequence, a potential constituent segment of the binding sites for κ-bungarotoxin (κ-BGT) and κ-flavitoxin (κ-FTX) was mapped to the sequence region α3(51-70) (McLane et al., 1990a, 1993). κ-BGT binds to this sequence with a K_d of ~300 n*M*; α-BGT does not bind to any α3 peptides. The sequence α3(51-70) contains several negatively charged residues that may interact with the Lys and Arg residues present in the disulfide-stabilized sequence loops of κ-BGT believed to interact with the AChR. It also contains several aromatic amino acids, which are a consistent structural feature of the α-BGT, κ-BGT and κ-FTX binding prototypes.

Two other largely overlapping peptide sequences that bind κ-BGT, α3(180-199) and α3(183-201), were identified using a competition assay with native neuronal AChR on PC-12 cells (most likely the α3/β2 subtype) (McLane et al., 1990a). Both peptides contain the vicinal Cys pair and are homologous to, although relatively divergent from, the muscle-type α-BGT binding sequence α181-200 of different species. Therefore, also in the α3 neuronal AChR, the sequence region surrounding the vicinal cysteines, which are the hallmark of the α-subunits, is likely to contribute to the cholinergic binding site. An involvement of this region of the α3 subunit in κ-BGT binding is supported by *Xenopus* oocyte expression studies using α2/α3 chimeras (Luetje and Patrick, 1991; Luetje et al., 1992). Thus, like α-BGT, κ-BGT appears to have multipoint attachments to the α3 subunit, and the segments of the α3 subunit contributing to the κ-BGT and κ-FTX binding site are homologous to those contributing to that α-BGT site in the *Torpedo* α-subunits.

2. α-Bungarotoxin-Sensitive Neuronal AChRs

Synthetic peptides have been used to obtain clues about the neurotoxin sensitivity of neuronal AChRs containing α-subunits not successfully expressed in *Xenopus* oocytes as functional complexes, such as the α5 subunit. It is unclear if failure to demonstrate α-BGT binding to *Xenopus* ooxytes injected with α5 transcripts is the result of low levels of expression (Sumikawa and Gehle, 1992), failure of assembly of complexes and/or membrane insertion, or if other unidentified subunits are required for functional expression (Boulter et al., 1990a; Couturier et al., 1990a). However, α5 mRNA expression correlates directly with the expression of neuronal α-BGT binding AChRs in several cell lines (Chini et al., 1992).

Overlapping peptides corresponding to the sequence region 171-205 of the α5 subunit, and of the mouse muscle α1 and rat neuronal α2, α3, and α4 subunits, which all contain the vicinal Cys_{192} and Cys_{193}, were compared for their ability to bind α-BGT. Peptides corresponding to this region of the *Torpedo* α-subunit (Conti-Tronconi et al., 1990a) and the muscle α-subunits of a number of species (McLane et al., 1991a) form prototopes for α-BGT (see Section VIII.A). In a solid-phase assay testing the direct binding of ^{125}I-α-BGT to synthetic peptides, and in two different competition assays, in which peptides were tested for their ability to sequester ^{125}I-α-BGT from binding to native AChR on postsynaptic membrane fragments of *Torpedo* or electric organ or PC-12 cells, only peptides corresponding to the mouse muscle α1 and rat neuronal α5 subunits bound α-BGT (McLane et al., 1990b). These results are consistent with the known pharmacology of the α1, α2, α3, and α4 AChR subtypes, and suggest that the α5 subunit is able to bind α-BGT. The most prominent α-BGT-binding peptide sequence of the α5 subunit was α5 (180-199).

Synthetic peptide mapping studies of α-BGT binding regions on the complete sequence of the chick brain α7 subunit, and on selected sequence regions of the highly homologous α8 subunit, were also carried out (McLane et al., 1991c). The cDNAs for these neuronal α-subunits were isolated using oligonucleotides corresponding to the amino-terminal sequence region of an α-subunit from AChR proteins isolated from chick brain using α-BGT affinity chromatography (Conti-Tronconi et al., 1985; Schoepfer et al., 1990). Peptides corresponding to the sequence segments α7(181-200) and α8(181-200) bound α-BGT with

affinities similar to those reported for the muscle AChR α-subunits (McLane et al., 1991a). The ability of the α7 and α8 subunit peptides to bind α-BGT was surprising, given their highly divergent sequences relative to the muscle α-subunits. These results have been confirmed by expression studies that indicate that AChR homomeric complexes formed by the α7 subunit are blocked by α-BGT (Couturier et al., 1990b; Bertrand et al., 1992).

The peptide studies of the α5, α7, and α8 α-BGT binding sites illustrate how the synthetic peptide approach can be used to predict the potential pharmacology of an α-subunit subtype prior to its functional reconstitution.

C. Identification of Individual Residues Interacting with α-Bungarotoxin: Comparison of Homologous Sequences

1. Sequences α(181-200) from Muscle AChR of Different Species

Naturally occurring species-specific amino acid substitutions provided a first step toward identification of individual residues important for α-BGT binding. Muscle α-subunits are highly conserved between different species, and all contain an α-BGT-binding prototype within amino acid residues α181-200, with the notable exceptions of the α-subunits of snake species resistant to the blocking action of α-BGT (e.g., cobra, natrix) (Ohana and Gershoni, 1990; McLane et al., 1991a; Ohana et al., 1991; Fuchs et al., 1993), and of the mongoose, a mammalian resistant to snake neurotoxins (Ovadia and Kochva, 1977), well known for its ability to kill and eat poisonous snakes (e.g., see Kipling, 1895) (Figure 2). Six amino acid residues of this sequence region in the snake α-subunit differ from the other α-subunits and may represent important residues for α-BGT binding. Nonconservative substitutions in the snake α181-200 sequence include replacements of Lys_{185}, Trp_{187}, Tyr_{189}, and Pro_{194} by Trp, Ser, Asn, and Leu, respectively. Single-residue mutations of the *Torpedo* α-sequence to each of the six substitutions of the cobra α-sequence demonstrated that conversion of Tyr_{189} to Asn or Pro_{194} to Leu in the *Torpedo* sequence suffices to

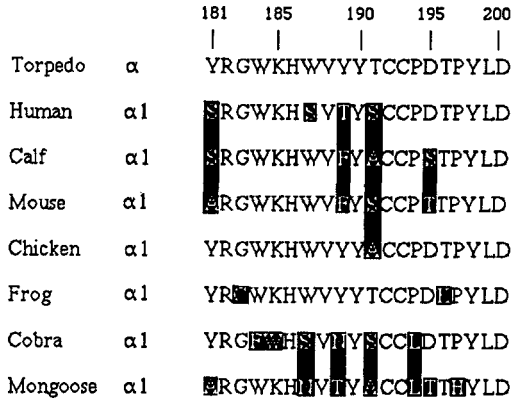

FIGURE 2. Alignment of the sequence region 181-200 from the α subunit of different vertebrate muscle AChRs. The amino acid residues that are substituted, when compared with the *Torpedo* α subunit, are boxed. This sequence region is highly conserved in most muscle α subunits. Several nonconservative substitutions within this region are present in the α subunits of cobra and mongoose muscle AChRs. This may be related to the resistance of muscle from these species to α-neurotoxin block, and the inability of synthetic peptides corresponding to the cobra and mongoose sequence region α181-200 to bind α-BTX. See text for further details.

eliminate α-BGT binding (Ohana et al., 1991; Chaturvedi et al., 1992). In the mongoose sequence, Trp_{187}, Tyr_{189}, and Pro_{194} are non-conservatively substituted to Asn, Thr, and Leu, respectively (Fuchs et al., 1993). In addition, the invariant Pro_{197}, conserved in all known peripheral AChR α-subunits, is nonconservatively substituted to His, and at position 195, the negatively charged residue present on most α-BGT-binding prototopes and proposed to be involved in interaction with α-BGT (Tzartos and Remoundos, 1990), is conservatively substituted by Thr.

Comparison of the vertebrate muscle α-sequences (Figure 2) shows that the sequence Val-Val-Tyr at positions 188-190 is common to the peptides that bind α-BGT with high affinity (*Torpedo*, frog, and chick), and that substitution of amino acids at position 189 to Phe (calf and mouse sequences), Asn (cobra sequence), or Thr (human and mongoose sequence) correlates with a reduced affinity of this sequence

region for α-BGT (McLane et al., 1990a). The convergence of these results strongly indicates Tyr_{189} as a critical residue in the interaction of α-subunits from different muscle AChRs with α-BGT.

2. Sequences α(181-200) from Neuronal AChRs of Different Species and Subtypes

The sequence region surrounding the vicinal cysteines 192/193 of the neuronal α-BGT binding α-subunits is highly diverged with respect to the *Torpedo* and muscle α1 subunits (Figure 3). The low predictive value of sequence homology to infer neurotoxin sensitivity can be best appreciated by comparison of the sequence regions of AChR α-subunits that bind α-BGT (*Torpedo* α, vertebrate muscle α1, rat neuronal α5, chick brain α7 and α8, and *Drosophila* ALS and SAD subunits) (Figure 3, top panel), with the homologous sequence regions of AChRs that do not bind α-BGT (the cobra and mongoose muscle α1, and the neuronal α2, α3, and α4 subunits) (Figure 3, bottom panel). Seven amino acid residues are characteristic of all α-subunits regardless of their α-BGT binding ability: Gly_{183} (or the conservative substitution Ala), Tyr_{190}, Cys_{192}, Cys_{193}, Asp_{195} (or the conservative substitution Glu), Tyr_{198}, and Asp_{200}. All of the α-subunits in Figure 3 that bind α-BGT have Tyr_{189} (or the conservative substitution Phe) and Pro_{197}, whereas Lys_{189} and Ile_{197} are characteristic of α-subunits that do not bind α-BGT. This general rule, however, does not hold for the α5 subunit sequence, which is highly divergent from other AChR α-subunits and offers a unique opportunity to study the structural requirements for α-BGT binding. The inability to correlate critical structural features required for α-BGT binding with a particular amino acid sequence indicates, in a broader sense, a serious limitation to the use of sequence homology to define families of functionally and structurally related proteins. It is obvious from comparison of the α-BGT binding sequences that different primary sequences must fold into three-dimensional structures with comparable hydrophobic, hydrogen-bonding, and charge interactions. Compensatory,

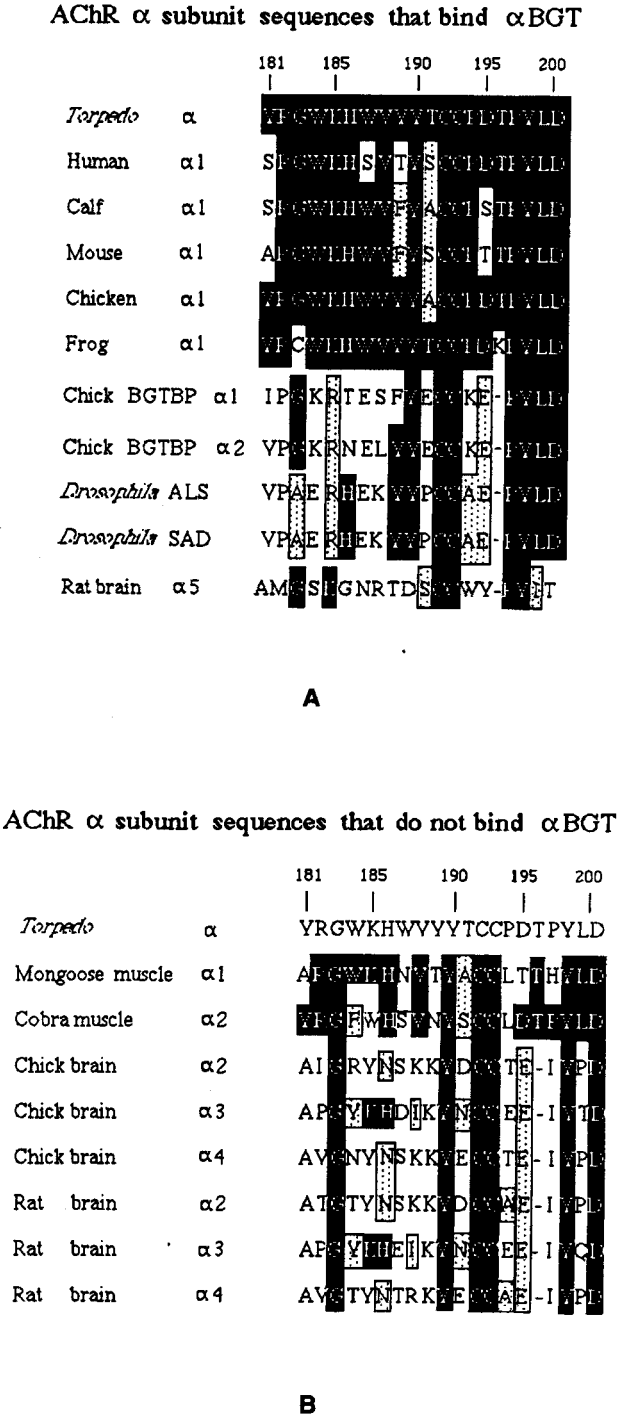

FIGURE 3. Alignment of the sequence region 181-200 from the α subunit of different muscle and neuronal AChRs. The residue are numbered with reference to the *Torpedo* α subunit sequence. Identical residues (when compared with the *Torpedo* sequence, which, for sake of comparison, is also aligned at the top of the sequences that cannot bind α-BTX) are enclosed in black boxes, conservative substitutions in dotted boxes. See text for further details.

multiple nonconservative substitutions that occurred during the evolution of α-BGT binding proteins have obscured a "universal" α-BGT binding motif. This fact is also illustrated by the lack of sequence homology between any of the nicotinic AChR α-subunits and the ACh binding sites of the muscarinic ACh receptor and acetylcholinesterase (Schumacher et al., 1986; Hulme et al., 1990). The failure to find a common α-BGT binding motif is similar to the search for targeting sequence signals involved in sorting proteins into different cellular compartments. In those cases, instead of primary sequence conservation, compositional motifs are found in which certain amino acids or residues with similar physical characteristics are common among proteins destined to the same cellular organelle or membrane compartment (e.g., see Dice, 1990).

D. Individual Residues Interacting with α-Bungarotoxin, κ-Bungarotoxin, and κ-Flavitoxin: Studies with Single-Residue-Substituted Peptide Analogs

Given the lack of sequence motifs identifying α-BGT binding proteins, mutational analysis has been used in attempts to determine the structural requirements for α-BGT binding.

1. Torpedo *and Muscle* α*-Subunit Sequences*

Substituted peptide analogs of the *Torpedo* sequence α181-200 have been used with single amino acid substitutions of glycine for each native residue (Conti-Tronconi et al., 1991). CD spectral analysis indicated that the substituted analogs had comparable structure. However, they differed in their ^{125}I-α-BGT binding activity. These studies identified distinct clusters of amino acid residues, discontinuously positioned along the sequence 181-200, which are critical for α-BGT binding and should serve as attachment points for α-BGT (residues 188-190 [Val-Tyr-Tyr] and 192-194 [Cys-Cys-Phe]).

Tzartos and Remoundos (1990) used overlapping peptides to define the minimum α-BGT binding sequence as *Torpedo* α188-197. Single amino acid substitutions (to Gly or Ala) of this sequence segment indicated that Tyr_{189}, Tyr_{190}, and Asp_{195} are important for α-BGT binding.

Chaturvedi et al., (1992) tested the effect of multiple amino acid substitutions of the *Torpedo* sequence α166-211 expressed as a bacterial fusion protein. Several substitutions were administered, based on nonconserved residues of the rat neuronal α3 and cobra muscle α1 subunits, neither of which bind α-BGT: (1) substitution of Trp_{184}, Lys_{185}, and Trp_{187} to Phe, Trp, and Ser, respectively (as found in the cobra α1 subunit) had no effect on α-BGT binding, while introduction of two more mutations, Thr_{191} to Ser and Pro_{194} to Leu, abolished α-BGT binding, (2) single amino acid residue mutations of the *Torpedo* sequence to cobra α1 residues, Pro_{194} to Leu or Tyr_{189} to Asn, abolished α-BGT binding, and (3) mutation of the *Torpedo* sequence with three substitutions found in the α3 subunit (Trp_{187}, Tyr_{189}, and Thr_{191} to Asp, Lys, and Asn, respectively) eliminated α-BGT binding activity. These results confirmed that Tyr_{189} and Pro_{194} are critical for α-BGT binding.

Several mouse muscle α-subunit mutants expressed as native AChR complexes in *Xenopus* oocytes were tested for α-BGT binding (Tomaselli et al., 1991). None of the following mutations affected the affinity of α-BGT binding: His_{186} to Phe, Tyr_{190} to Phe, Pro_{194} to Ser, and Tyr_{198} to Phe. On the other hand, the affinity for ACh was markedly reduced when Tyr_{190} was substituted to Phe, suggesting that α-BGT and ACh have different structural requirements for binding. The different results obtained when using synthetic and biosynthetic mutated sequences of *Torpedo* and mouse α-subunits, and when using AChR complexes mutated at individual residues could be because of species differences, to differences in the amino acid substitutions made, or, most likely, to the fact that binding of α-BGT to native AChR occurs via large interacting surfaces, and mutation of one of the several residues involved in such an interaction may not suffice to change the binding affinity detectably.

2. Neuronal AChR α*-Bungarotoxin Binding Sequences*

The α-BGT binding sequence region 180-200 of the rat α5 subunit is relatively divergent

when compared with the homologous sequence regions of *Torpedo* and muscle AChRs (see Section VIII.B.2). Amino acid residues critical for α-BGT binding were identified by testing the effects of single amino acid substitutions to Gly or Ala for each residue of the rat α5(180-199) sequence on binding of α-BGT to the substituted peptide analogs (McLane et al., 1991d). Substitutions of four residues (Lys_{184}, Arg_{187}, Cys_{191}, and Pro_{195}) abolished α-BGT binding; other substitutions (Gly_{185}, Asn_{186}, Asp_{189}, Trp_{193}, Tyr_{193}, and Tyr_{196}) lowered its affinity. The importance of several aromatic amino acids for α-BGT binding to the α5 peptide is analogous to the findings reported above for the *Torpedo* α180-200 sequence. Thus, despite the apparent divergence of the α5 sequence from other α-BGT-binding α-subunits, some structural features, such as an abundance of aromatic residues and amino acids able to contribute electrostatic and/or hydrogen bond interactions, have been conserved.

3. Neuronal AChR κ-Toxin Binding Sequences

The sequence segment α3(50-71) forms a prototope for κ-BGT and κ-FTX in the rat α3 subunit (McLane et al., 1990a, 1993). Synthetic peptide analogs of the sequence α3(50-71), in which each amino acid was sequentially replaced by Gly, were tested for their ability to bind ^{125}I-κ-BGT and ^{125}I-κ-FTX (McLane et al., 1991b, 1993). No single substitution obliterated κ-BGT binding, but several substitutions lowered the affinity of this peptide sequence for κ-BGT — two negatively charged residues (Glu_{51} and Asp_{62}) and several aliphatic and aromatic residues (Leu_{54}, Leu_{56}, and Tyr_{63}). Similar to κ-BGT, aliphatic and aromatic amino acid residues were important for κ-FTX binding (Leu_{54}, Leu_{56}, and Tyr_{63}, also involved in κ-BGT binding, and additional Trp residues at positions 55, 60, and 67). In contrast to κ-BGT, however, positively rather than negatively charged amino acids appeared to mediate electrostatic interactions with κ-FTX — Lys residues at positions 57, 64, 66, and 68. These differences in amino acid specificity can be correlated with sequence differences of κ-BGT and κ-FTX, and provide clues as to the residue interactions at the κ-toxin subunit interface (McLane et al., 1993).

E. A Welcome Summary of the Results of Studies on the Structure of Cholinergic Binding Sites by the Use of Synthetic or Biosynthetic Peptides

The studies summarized in the previous sections allow the following conclusions:

1. Two or three sequence segments of the α-subunit contribute to form the cholinergic binding sites recognized by snake α- and κ-neurotoxins (α-BGT, κ-BGT, and κ-FTX). These sites therefore are complex surface areas, formed by clusters of amino acid residues from different sequence regions, similar to the "discontinous epitopes" of antibody-antigen complexes (Davies et al., 1988).
2. In all the AChRs studied, the sequence segments contributing to the cholinergic site are in similar positions along the α-subunit sequence, suggesting that the extracellular domain of all α-subunits folds in a similar manner. The sequence segment containing Cys_{192}/Cys_{193} is part of a cholinergic site in all α-subunits.
3. In peripheral AChRs, the sequence region α180-200 is well conserved, and well-defined clusters of residues surrounding and including the residues Cys_{192} and Cys_{193} are involved in interaction with α-BGT. The corresponding sequence region is not well conserved in the neuronal AChRs that bind α-BGT, and the residues identified as crucial for interaction with α-BGT are at positions different than those of peripheral AChRs. Therefore, there is no universal sequence motif with predictive value for α-BGT binding, and multiple, nonconservative substitutions in these sequence regions that occurred during evolution of the AChR proteins have both obscured the original ancestral sequence and reestablished, as a result of new mutual interactions, a structure compatible with α-BGT binding.
4. Although Cys_{192}/Cys_{193} are involved in forming the toxin/α-subunit interface, a vicinal disulfide bond is not required for α-BGT binding.

5. Within the relatively large area forming the cholinergic site, cholinergic ligands bind with multiple points of attachment, and ligand-specific patterns of attachment points exist. This may be the molecular basis of the broad spectra of binding affinities, kinetic parameters, and pharmacologic properties observed for the different cholinergic ligands.
6. The sequence regions α181-200 and α50-75 are unusually rich in aromatic residues, and substitution of aromatic residues frequently abrogates or decreases α-BGT (or κ-BGT or κ-FTX) binding. These findings are compatible with the suggested model for the anionic cholinergic binding site of the AChR as formed not by a single negatively charged residue, but rather by interaction of the π-electrons of aromatic rings (Dougherty and Stauffer, 1990), as has been demonstrated for the cholinergic site of acetylcholinesterase (Sussman et al., 1991) (discussed in Section VIII).

IX. ATOMIC STRUCTURE OF THE ACh BINDING SITE OF ACETYLCHOLINESTERASE: IMPLICATIONS FOR STRUCTURAL REQUIREMENTS OF THE CHOLINERGIC SITE OF THE AChR

The binding site for acetylcholinesterase has been well characterized through the use of different types of inhibitors. It has been proposed that an anionic site within the enzymatic pocket of acetylcholinesterase stabilizes binding of the quaternary ammonium group of ACh. However, the existence of a charged site is incompatible with the high-affinity binding of neutral analogs of ACh (Dougherty and Stauffer, 1990, and references therein). Photoaffinity labeling of acetylcholinesterases from different sources consistently identified aromatic amino acids as contributing to the active site, whereas no charged residues have been found (Dougherty and Stauffer, 1990). Based on these findings, it was suggested that a binding site containing aromatic amino acid residues could stabilize the binding of the quaternary ammonium group through cation/π-electron interactions (Dougherty and Stauffer, 1990). Using a synthetic receptor composed of aromatic rings, it was demonstrated that a π-electron-rich system can indeed bind the positive charge of ACh.

More recently, the atomic structure of acetylcholinesterase has been determined (Sussman et al., 1991). The catalytic triad of the enzyme Ser-His-Glu lies at the end of a gorge lined with ~14 aromatic residues. Thus, the predictions that aromatic residues may form a binding site for ACh and that cation/π-electron interactions stabilize the binding of the quaternary ammonium group have been fulfilled.

These results relate to studies of the effects on the binding of ACh (Tomaselli et al., 1991) and α- and κ-neurotoxins of amino acid substitutions within sequence regions of different AChR α-subunits likely to contribute to the formation of a binding site for ACh and α-BGT (regions α181-200 and α50-75; see Section VIII). Those studies consistently demonstrated that aromatic residues are abundant in this region and are important for ligand binding. Furthermore, aromatic residues are frequently labeled by cholinergic affinity labels (see Section VII.B). It is likely that, as in the binding site of acetylcholinesterase, in the AChR π-electrons contributed by several aromatic rings, rather than a single negatively charged residue, stabilize the positive charge of ACh and other cholinergic ligands.

X. SUBUNITS OTHER THAN THE α ARE INVOLVED IN THE FORMATION OF CHOLINERGIC SITES, WHICH APPEAR TO BE AT SUBUNIT/SUBUNIT INTERFACES

A vast body of information on the binding of peptide neurotoxins to AChRs has been reviewed previously (e.g., see Katz and Miledi, 1971; Land et al., 1981; Adams, 1981; Conti-Tronconi and Raftery, 1982; Karlin et al., 1986; Lentz and Wilson, 1988; Claudio, 1989; Stroud et al., 1990; Galzi et al., 1991). α-BGT has been of major importance in the isolation and characterization of peripheral AChRs and certain neuronal AChRs. In *Torpedo* AChR, α-BGT binds to two sites per AChR molecule. For membrane-bound AChR, these sites have different affinities (Conti-Tronconi

et al., 1990b). One has very high affinity, and the complex dissociates with a half-life of ~250 h (Conti-Tronconi and Raftery, 1986); the other has lower affinity, and the complex is readily reversible (Conti-Tronconi et al., 1990b). Other neurotoxins, including α-cobrotoxin (α-NTX) from *Naja naja siamensis* (Conti-Tronconi and Raftery, 1986) and the coral neurotoxin, lophotoxin (Culver et al., 1984), also bind to two sites on the AChR. By contrast, α-dendrotoxin (α-DTX) from *Dendroaspis viridis* binds to the membrane-bound *Torpedo* AChR with a stoichiometry of four toxins per receptor molecule (Conti-Tronconi and Raftery, 1986). Furthermore, when α-DTX binds to preformed AChR/α-BGT complexes, the rate of dissociation of α-BGT is increased from a $t_{1/2}$ of 250 h to 4.5 h, suggesting that there are cooperative interactions between the additional sites for α-DTX and the sites for α-BGT (Conti-Tronconi and Raftery, 1986).

A. Use of Peptide Neurotoxins as Affinity Labels and as Probes of Cholinergic Site Stoichiometry

Snake α-neurotoxins have been used to affinity label AChR (reviewed in Lentz and Wilson, 1988). In all studies using native or derivatized α-BGT, cross-linking to the α-subunits was observed. This has been confirmed in several studies in which α-BGT was shown to bind to isolated α-subunits, albeit with lower affinity (reviewed in Lentz and Wilson, 1988). α-BGT labeling of other *Torpedo* AChR subunits was also observed. In some studies, α-BGT cross-linked the α- and δ-subunits (Witzemann and Raftery, 1979); Witzemann et al., 1979). In another study, α-BGT photolabeled the α-, γ-, and δ-subunits (Oswald and Changeux, 1982). These results have been interpreted to indicate that the α-neurotoxins bind mainly to the α-subunits, with perhaps some overlap with respect to adjacent subunits. However, α-BGT labeling of subunits other than the α-, and of the γ- and δ-subunits in particular, may indicate that the two sites for α-BGT are at interfaces between subunits, and in particular at the α/γ and α/δ interfaces.

Some α-conotoxins block AChR function (Olivera et al., 1990). Cross-linking of AChR by derivatized α-conotoxins or photoaffinity labeling covalently labeled the β- and γ-subunits, with little or no labeling of the α- or δ-subunits (Myers et al., 1991). These results clearly demonstrate the importance of subunits other than the α in ligand binding site formation. The γ- and δ-subunits have also been implicated in forming at least part of the binding sites for ^{3}H-tubocurare (Blount and Merlie, 1989; Pederson and Cohen, 1990) and ^{3}H-nicotine (Middleton and Cohen, 1991), and it has been suggested that binding sites for these ligands lie at the α/γ and α/δ subunit interfaces (see Section X.B and C).

B. Expression Studies Indicate that Subunits Other Than the α Contribute to the Formation of Cholinergic Sites

For all AChR subunits studied so far, expression of the α-subunit is necessary to reproduce ligand binding activity (reviewed in Claudio, 1989). For reconstitution of a functional AChR, however, other subunits are needed (Blount and Merlie, 1989; Kurosaki et al., 1987), with the exception of the vertebrate neuronal α7 subunit (Couturier et al., 1990b) and α-subunits from insect neurons (see Section III.B). Experiments testing expression of different types of neuronal β-subunits in association with the same α-subunit indicated that the β-subunit influences the pharmacologic properties of the resulting AChRs (Goldman et al., 1987; Wada et al., 1988; Duvoisin et al., 1989; Luetje et al., 1990a, b) — for example, κ-BGT blocks AChR formed by coexpression of α3 and β2 subunits, but not α3 and β4 subunits (Duvoisin et al., 1989; Luetje et al., 1990a, b). In muscle AChRs, coexpression of α- plus γ- and/or δ-subunits is necessary for δ-tubocurare binding, and expression of α/γ or α/δ subunit pairs determines the binding affinity for δ-tubocurare (Blount and Merlie, 1989; Blount et al., 1990; Gu et al., 1991; Sine and Claudio, 1991). Thus, these two subunit pairs well account for the two nonequivalent δ-tubocurare binding sites of native AChR, which must be at the subunit/subunit interface.

Cooperativity of agonist binding is also a function of intersubunit interactions. Expression of triplets of muscle AChR subunits on the sur-

face of mammalian cells demonstrated that cooperative agonist binding is lost if the γ- or δ-subunits are not coexpressed with the α-subunit (Sine and Claudio, 1991).

C. Labeling of α/γ and α/δ Subunit Pairs with Affinity Labels

Affinity photolabeling of *Torpedo* AChR with ^{3}H-δ-tubocurare further proved that the two nonequivalent δ-tubocurare binding sites are at the α/γ and α/δ subunit interfaces. The α-, γ-, and δ-subunits were specifically labeled, with dose dependence consistent with a high-affinity site at the α/γ and low-affinity site at the α/δ interface (Pedersen and Cohen, 1990). Photoaffinity labeling with ^{3}H-nicotine verified this interpretation, further indicating that cholinergic sites are located at the α/γ and α/δ interfaces (Middleton and Cohen, 1991). Formation of binding sites at the interfaces of two subunit domains follows the same structural motif as several active sites of enzymes.

Using particular conditions for disulfide bond reduction, ^{3}H-BAC labels both α- and γ-subunits (Dunn et al., 1993), further implicating the γ-subunit as contributing to an agonist binding site. An earlier study also suggested that the γ-subunit was in close proximity to the binding site for the cholinergic affinity label dimethylamino-benzenediazonium fluoroborate (DDF), which is incorporated into the γ-subunit, as well as the α-subunit (Dennis et al., 1988).

The involvement of the δ-subunit in forming a cholinergic binding site is supported by the cross-linking of Cys_{192}/Cys_{193} with a carboxylate group within the sequence segment δ164-224 (Czajkowski and Karlin, 1991). Another conclusion from that study was that a carboxylate group from Asp and/or Glu residue(s) of the δ-subunit could stabilize the binding of the quaternary ammonium group of ACh bound to the α-subunit in the vicinity of (actually ~9 Å away) the region containing Cys_{192}/Cys_{193}. The requirement for a negatively charged group for stabilizing the quaternary ammonium ion of ACh, however, is currently being questioned, as discussed in Section IX.

D. Differential Protection by Cholinergic Ligands of the Labeling of Particular Subunit Pairs by DAPA, a Cholinergic Affinity Ligand Able to Label all AChR Subunits

^{3}H-DAPA, a cholinergic affinity label, labels multiple AChR subunits in an α-BGT-sensitive manner (Witzemann and Raftery, 1977). Recent results have shown that all four *Torpedo* AChR subunits are labeled by ^{3}H-DAPA (Tine and Raftery, 1993). Labeling of the α-subunits was the least sensitive to inhibition by cholinergic agonists. Furthermore, if AChR was previously saturated by covalent labeling of the α-subunits by BAC (2 mol/mol AChR), labeling of all subunits except α was inhibited. These findings suggest that the α-subunits contain binding sites for cholinergic ligands unaffected by covalently bound BAC.

XI. ANTIBODIES TO MUSCLE AChR ARE THE EFFECTORS OF MG SYMPTOMS

A. Anti-AChR Antibodies Are Polyclonal High-Affinity IgGs

MG symptoms can be transferred to healthy animals, or reproduced *in vitro* with nerve/muscle preparations, by administering the IgG fraction from the sera of MG patients, or polyclonal or monoclonal anti-AChR antibodies from animals that developed experimental autoimmune MG (EAMG) (Toyka et al., 1975; Lindstrom et al., 1976; Berti et al., 1976; Richman et al., 1980; Lennon and Lambert, 1980; Tzartos et al., 1987). Anti-AChR antibodies are likely to be the only effectors of MG symptoms, because no cytotoxic phenomena have been demonstrated in MG patients (Engel, 1984; Levinson et al., 1988; Lindstrom et al., 1988; Schönbeck et al., 1990). Antibodies against non-AChR muscle or synaptic proteins are present in sera from myasthenic patients. Some such antibodies (e.g., antibodies against a presynaptic membrane protein that bind β-bungarotoxin [Lu et al., 1991] or a calcium-releasing sarcoplasmic membrane protein

[Mygland et al., 1992]) may cause myasthenic symptoms, especially in MG cases without measurable anti-AChR antibodies (see below).

Anti-AChR antibodies from MG patients seldom recognize and block the cholinergic binding site (reviewed in Engel, 1984; Levinson et al., 1988; Lindstrom et al., 1988; Schönbeck et al., 1990). They cause increased AChR destruction either by causing accelerated degradation of the AChR, triggered by antibody cross-linking of two nearby AChR molecules (antigenic modulation), or by complement activation and complement-mediated lysis of the postsynaptic membrane (reviewed in Engel, 1984; Levinson et al., 1988; Lindstrom et al., 1988; Schönbeck et al., 1990). The postsynaptic membrane at the neuromuscular junction of MG patients is impoverished in AChR and has a simplified structure, without the normal postsynaptic folding (Engel et al., 1981; Engel and Santa, 1971). The intersynaptic space contains immunoglobulins and complement (Sahash et al., 1980).

Anti-AChR antibodies in MG patients are polyclonal (reviewed in Engel, 1984; Levinson et al., 1988; Lindstrom et al., 1988; Schönbeck et al., 1990), and even antibodies against an individual AChR epitope do not have preferential idiotype(s) (Killen et al., 1985; Dwyer et al., 1984). Therefore, MG is not caused by the expansion of a single "forbidden" B-cell clone. Although anti-AChR antibodies are the hallmark of MG, there is very poor or no correlation between antibody titer and severity of the disease (Engel, 1984; Levinson et al., 1988; Lindstrom et al., 1988; Schönbeck et al., 1990; Roses et al., 1981; Besinger et al., 1983). This suggests that only particular subpopulations of anti-AChR antibodies are pathogenetic, perhaps because they are better able to cause antigenic modulation and/or complement activation. In support of this possibility, a study using different monoclonal antibodies against the AChR α-subunit demonstrated that few of them cross-linked AChR molecules and induced antigen modulation, perhaps because of the geometry of the epitope location on the AChR surface (Conti-Tronconi et al., 1981).

Some MG patients do not have detectable anti-AChR antibodies (Mossman, 1986; Evoli et al., 1989). They may produce antibodies against non-AChR components of the neuromuscular junction (see above) because their symptoms frequently improve after plasmapheresis (Miller et al., 1981), and injection into mice of their immunoglobulins causes impairment of neuromuscular transmission without detectable antibodies bound to mouse AChR (Schönbeck et al., 1990).

B. The Main Immunogenic Region (MIR), a Small Extracellular Area of Muscle AChR, Dominates the Anti-AChR Antibody Response

Most anti-AChR antibodies in the sera of rats that, after immunization with AChR develop EAMG, recognize a small extracellular region of the α-subunit, called the main immunogenic region (MIR) (reviewed in Lindstrom et al., 1988; Schönbeck et al., 1990). The MIR is highly conserved in AChRs from different species, and anti-MIR antibodies are highly cross-reactive (Tzartos and Lindstrom, 1980; Tzartos et al., 1982). Anti-MIR antibodies have a high pathogenetic potential, because their addition to muscle cell cultures causes AChR loss (Conti-Tronconi et al., 1981) and their injection into rats causes myasthenic symptoms (Tzartos et al., 1987).

A single anti-MIR monoclonal antibody inhibits an average of ~60% of the binding of the anti-AChR antibodies in the sera of MG patients, strongly suggesting that this epitope — or this set of largely overlapping epitopes — also dominates the antibody response in human MG (Tzartos et al., 1982). Anti-MIR antibodies are primarily responsible for the ability of the sera from MG patients to cause AChR loss in cell cultures (Tzartos et al., 1985).

The existence of the MIR as the immunodominant B-cell epitope in MG has been questioned by one study that proposed that anti-MIR antibodies inhibit binding to AChR of the antibodies in MG sera unspecifically because of allosteric hindrance (Lennon and Griesman, 1989).

It is not known why the MIR dominates the anti-AChR antibody response. T- and B-cell immunodominance of a relatively small number of epitopes on a protein antigen in a given animal or inbred strain is a common occurrence. The

MIR may represent an extreme case of "focused" antibody response. However, the MIR is immunodominant in patients of different major histocompatiblity complex (MHC) haplotype and in different species, such as humans and rats (Lindstrom et al., 1988), suggesting that the MIR immunodominance could be related to structural features that make it more antigenic for B-cells, or result from antibody cross-reactivity with epitope(s) on microbial or autologous antigens. The AChR sequence α65-80, which contains the MIR (see below), is very similar to a sequence region of the U1 small nuclear ribonucleoprotein — one of the key autoantigens in systemic lupus erythematosus — and to a region of the pol-polyprotein of several human retroviruses (Manfredi et al., 1991).

C. Sequence Mapping of Constituent Elements of the MIR

The MIR is a conformational determinant, and many anti-MIR antibodies do not bind to denatured AChR (Tzartos et al., 1981; Conti-Tronconi et al., 1981; Tzartos and Lindstrom, 1980). However, some anti-MIR antibodies recognize the denatured AChR α-subunit, albeit with lower affinity than native AChR (Tzartos et al., 1988, 1990; Barkas et al., 1989; Bellone et al., 1989; Das and Lindstrom, 1989). This allowed the use of synthetic peptides to identify sequence regions and individual residues involved in MIR formation.

A sequence region contributing important constituent elements of the MIR has been localized within residues 67-76 of both the human and the *Torpedo* AChR α-subunits. Synthetic peptides containing this sequence segment consistently bound anti-MIR monoclonal antibodies (Tzartos et al., 1988; Barkas et al., 1989; Bellone et al., 1989). Another study attempted MIR localization by the use of biosynthetic α-subunit fragments, and restricted MIR localization to residues 61-70 of the *Torpedo* AChR α-subunit (Tzartos et al., 1990). Another study, which also utilized synthetic peptides, localized constituent elements of the MIR to residues 66-76 of the human muscle α-subunit (Das and Lindstrom, 1989). The manner of binding of different anti-MIR monoclonal antibodies to synthetic peptides corresponding to the sequence α67-76 plus different lengths of the flanking sequences indicated that the MIR contains different, largely overlapping epitopes (Tzartos et al., 1990).

Although the structure of the MIR is highly conserved, anti-MIR antibodies cross-react with AChRs of different species with different affinities (Tzartos and Lindstrom, 1980; Tzartos et al., 1982). Anti-MIR antibodies recognize synthetic peptides containing the sequence α67-76 from different animal species with the specificity corresponding to that displayed when bound to the native form of the corresponding AChRs (Tzartos et al., 1988; Barkas et al., 1988), supporting the possibility that the segment α67-76 indeed contains crucial structural components of the MIR. Comparison of the ability of different anti-MIR monoclonal antibodies to bind the synthetic sequence α67-76 of human muscle and *Torpedo* AChRs indicated that the residues at position 70 and 75, which are different in those AChRs, determine the binding preference of the antibody to the synthetic MIR sequence (Tzartos et al., 1990a).

Structural predictions suggest that the sequence region α67-76 in both *Torpedo* and human muscle AChRs is a hydrophilic hairpin loop, whose negatively charged apex is a type I β-turn formed by residues α68-71 and whose arms are β-strands (Bellone et al., 1989). Residues comprising the apex of this proposed loop are indeed involved in interaction with anti-MIR antibodies because studies of the binding of the anti-MIR monoclonal antibodies to single-residue-substituted analogs of this sequence indicated that residues α68-72, and most notably Asn_{68}, Pro_{69}, Asp_{71}, and Tyr_{72}, are crucial or important for anti-MIR antibody binding (Bellone et al., 1989). Substitutions of Asp_{70} of the human sequence with an Ala residue, as in *Torpedo* AChR, affected the binding of anti-human MIR antibodies, while it was irrelevant for the binding of other anti-MIR antibodies which, although cross-reacting with human AChR, were originally raised — and preferentially recognized — fish AChR.

That several amino acid residues are important for the binding of anti-MIR antibodies agrees with the accepted view that antibody epitopes include a relatively large area of protein anti-

gens, formed by several amino acid residues, and that antibody binding occurs through multipoint attachments (Davies et al., 1988).

The use of single-residue-substituted peptide analogs of the sequence α67-76 containing different conservative substitutions of residues previously identified as involved in anti-MIR antibody binding confirmed their role as important for MIR antigenicity and offered clues to its three-dimensional structure (Wahlsten et al., 1993). Conservative substitutions of residues Asn_{68} and Asp_{71} obliterated binding, identifying them as necessary for anti-MIR antibody interaction, whereas conservative substitutions at Asp_{70} and Tyr_{72} diminished binding only marginally. Identification of residues Asn_{68} and Asp_{71} of the α-subunit as crucial for MIR formation is supported by the fact that they are highly conserved in all known AChRs which bind anti-MIR antibodies, and nonconservatively substituted in a frog muscle AChR isomer, which is the only known muscle AChR unable to bind anti-MIR antibodies (Hartman and Claudio, 1990). A study that used synthetic peptides corresponding to the sequence region α67-76 of human and frog muscle verified that anti-MIR antibodies recognized only the human sequence, further suggesting that residues Arg_{68} and Asp_{71} are important for anti-MIR antibody binding (Das and Lindstrom, 1989). Furthermore, mutation of *Torpedo* AChR at these positions obliterates its ability to bind anti-MIR antibodies (Saedi et al., 1990).

A study which employed synthetic peptide analogs carrying different substitutions of the sequence α67-76 of *Torpedo* and human muscle AChRs confirmed that amino acids critical for anti-MIR antibody binding are within the segment α67-71; among them, Asn_{68} and Asp_{71} were crucial for binding of all the antibodies tested (Papodouli et al., 1990).

Some anti-MIR antibodies also bind a synthetic peptide corresponding to the sequence region α55-74 of *Torpedo* AChR, which includes the amino terminal of the sequence segment α67-76 (Tzartos et al., 1990). The use of single-residue-substituted synthetic analogs of the *Torpedo* sequence α55-74 identified further amino acids involved in MIR formation. Substitution of Trp_{60} and Asp_{62} affected the binding of some anti-MIR monoclonal antibodies, suggesting that these residues may be at the periphery of the MIR area (Wahlsten et al., 1993).

Many MG patients have low titers of anti-AChR antibodies that recognize the cholinergic site, as determined by their ability to block α-BGT binding (Engel, 1984; Levinson et al., 1987). Because residues from the sequence region α55-74, which is adjacent to the MIR, might contribute to formation of the cholinergic site (see Sections VIII.A and E), the MIR and α-BGT site may be in closer spatial proximity, on the AChR surface, than anticipated from the finding that α-BGT and anti-MIR antibodies are not mutually inhibitory (Tzartos and Lindstrom, 1980; Tzartos et al., 1981, 1983, 1985, 1987; Gullik and Lindstrom, 1983; Tzartos and Starzinski-Powitz, 1986). Studies on the binding of α-BGT and α-NTX to single-residue-substituted synthetic analogs of the sequence region α55-74 of *Torpedo* AChR concluded that Arg_{55}, Arg_{57}, Trp_{60}, Arg_{64}, Leu_{65}, Arg_{66}, Trp_{67}, and Asn_{68} are involved in the interaction with α-neurotoxins because their substitution drastically affected the binding of both α-BGT and α-NTX. Among these residues, only Asn_{68} is important for formation of the MIR, while Trp_{60} might be at the periphery of the MIR surface (see above). These results agree with a model where the MIR and the α-neurotoxin binding site, although spatially close, overlap minimally if at all (Tzartos and Lindstrom, 1980; Tzartos et al., 1981, 1983, 1985, 1987; Gullik and Lindstrom, 1983; Tzartos and Starzinski-Powitz, 1986).

XII. SEQUENCE REGIONS OF MUSCLE AChR RECOGNIZED BY ANTI-AChR Th CELLS

Anti-AChR antibodies in MG patients are high-affinity IgG, and their production requires the intervention of specific $CD4^+$ T-helper (Th) cells (Hohlfeld et al., 1984, 1986). An important role of anti-AChR Th cells in the pathogenesis of MG is supported by the findings that MG can be ameliorated by treatment with anti-CD4 antibodies (Waldor et al., 1983), and that the only obvi-

ous and early effect on the anti-AChR response of thymectomy — a staple in MG treatment — is a pronounced decrease in anti-AChR reactivity of circulating T-cells (Morgutti et al., 1979). The blood and thymus of MG patients contain anti-AChR $CD4^+$ Th cells that can be propagated *in vitro* by stimulation with AChR antigens (Hohlfeld et al., 1987, Melms et al., 1988; Harcourt et al., 1988; Protti et al., 1990, 1990a, 1991, 1991a; Manfredi et al., 1992, 1993; Moiola et al., submitted). They stimulate production of anti-AChR antibodies *in vitro* (Hohlfeld et al., 1986) and recognize the AChR in association with HLA-DR molecules (Hohlfeld et al., 1985; Manfredi et al., submitted; Moiola et al., submitted).

A. Experimental Approaches for Identification of the Epitope Repertoire of Anti-AChR Th Cells from MG Patients

Identification of the AChR sequence regions forming epitopes recognized by the autoimmune Th cells is important both for obvious theoretical considerations and for potential practical purposes, such as the design of specific immunosuppressive therapies.

Th cells recognize denatured, excised sequence segments of the antigen, in association with MHC class II molecules on the surface of antigen-presenting cells (APC) (Davis and Bjorkman, 1988; Ashwell and Schwartz, 1986). AChR-specific $CD4^+$ Th cells from the blood of MG patients recognize native or fully denatured AChR equally well (Hohlfeld et al., 1987). Therefore, biosynthetic and synthetic peptides corresponding to different subunits of the human AChR, whose sequence is known, can be used to propagate the autoimmune anti-AChR Th cells *in vitro* and identify the epitopes they recognize (see below). Biosynthetic AChR subunits, which can be produced with relative ease and in large amounts, are in principle ideal for propagation of subunit-specific Th lines (see below). On the other hand, relatively short peptides screening the sequences of the different AChR subunits are necessary to identify sequence regions forming T-epitopes. Synthetic peptides in principle could be processed differently than the cognate native antigen and result in epitopes different from those produced *in vivo*. The usefulness and legitimacy of the use of synthetic AChR sequences for the study of the anti-AChR $CD4^+$ response was verified by several studies on the response of unselected $CD4^+$ cells from the blood of MG patients to pools of synthetic peptides corresponding to the complete human muscle AChR α-, γ-, and δ-subunits (Protti et al., 1990; Manfredi et al., 1992, 1993). A $CD4^+$ response to the AChR peptides could be detected, whose intensity correlated with the severity of the disease (Protti et al., 1990; Manfredi et al., 1992, 1993). This relationship was particularly clear in long-term studies of the same MG patients. These results strongly support the possibility that the $CD4^+$ response detected by synthetic AChR sequences is involved in MG pathogenesis. Furthermore, as discussed below, $CD4^+$ cell lines propagated from MG patients by stimulation with synthetic AChR sequences respond vigorously to purified mammalian muscle AChR.

Identification of the epitope repertoire of anti-AChR Th cells has been pursued by testing the response to synthetic or biosynthetic AChR sequences of the total $CD4^+$ T-cells from the peripheral blood of MG patients, or of $CD4^+$ T-cell lines enriched in AChR-specific cells by *in vitro* culture with AChR antigens. These two approaches — use of unselected blood $CD4^+$ cells or of $CD4^+$ lines — have different advantages and caveats.

The use of unselected blood $CD4^+$ T-cells avoids detection of a biased repertoire due to selective clonal loss/enrichment during *in vitro* propagation of the lines. Studies that used the same panels of AChR synthetic sequences to test the response of AChR-specific $CD4^+$ cell lines (Protti et al., 1990a, 1991, 1991a, 1992), or unselected peripheral blood $CD4^+$ T cells (Manfredi et al., 1992, 1993) found that the $CD4^+$ blood cells responded far less vigorously than the $CD4^+$ lines, but to a larger repertoire of AChR epitopes. Because of the low frequency of AChR-specific Th cells in the blood (Sun et al., 1992), reliable testing of unselected peripheral blood $CD4^+$ T-cells is successful only in the most severely affected patients (Protti et al., 1990; Manfredi et al., 1992, 1993).

Long-term cell lines highly enriched in AChR-specific Th cells can be propagated *in vitro* from the blood or thymus of MG patients by cycles of stimulation with AChR antigens, such as purified AChR or synthetic or biosynthetic AChR sequences (reviewed in Protti et al., 1993). Polyclonal and monoclonal Th lines specific for one defined AChR epitope can also be obtained by stimulation with the corresponding synthetic or biosynthetic AChR sequence. Such lines allow studies of the structure of individual AChR epitopes and of their structural relationship with the specific T-cell receptors.

Successful propagation of AChR-specific long-term CD4+ cell lines was first obtained by cycles of stimulation with *Torpedo* AChR (Hohlfeld et al., 1984). Given the limited sequence similarity between *Torpedo* electric organ and human muscle AChRs (reviewed in Claudio, 1989), such lines should be biased toward epitopes formed by the most conserved sequence regions. In agreement with this prediction, the lines recognized preferentially or exclusively epitopes on the AChR α-subunit, which is most conserved among nonneuronal AChRs from different species. Synthetic AChR sequences have been used for propagation of anti-AChR T-cell lines. This approach has the important caveat that short synthetic sequences might be processed differently than the complete AChR molecule. Indeed, a study that used synthetic AChR sequences for propagation of CD4+ T-cell lines described lines that did not cross-react with native mammalian muscle AChR (Willcox et al., 1993). On the other hand, in several other studies, Th lines propagated from MG patients by stimulation with pools of synthetic AChR peptides responded vigorously to mammalian muscle AChR (Protti et al., 1991, 1992; Moiola et al., submitted). Taken together, these results indicate that the use of synthetic AChR antigens for propagation is legitimate, but caution needs to be exercised, and the AChR specificity of the lines must be verified by challenging them with the purified mammalian muscle AChR molecule before their further use for studies on the structure of autoimmune AChR epitopes.

The combined results of these approaches has identified many AChR sequence regions forming CD4+ epitopes, as discussed in the following sections.

B. Identification of a Large Th Repertoire on Each AChR Subunit

Most studies on the Th repertoire have focused on the AChR α-subunit, perhaps because of its importance in the anti-AChR antibody response (Schönbeck et al., 1990; Protti et al., 1990b; Manfredi et al., 1992; Oshima et al., 1990; Sommer et al., 1991; Brocke et al., 1989; Melms et al., 1989). Other studies have employed overlapping synthetic peptides corresponding to the complete sequence of β-, γ-, and δ-subunits to investigate whether these subunits are involved in the pathogenesis of MG and to identify sequence regions forming epitopes for CD4+ T-cells (Protti et al., 1991, 1991a, 1992; Manfredi et al., 1993; Moiola et al., 1994a). The several epitopes identified in those studies are summarized in Table 7.

Numerous sequence regions recognized by anti-AChR Th cells were identified on each subunit (Table 7). Several 20-residue regions were recognized by Th cells from most patients, irrespective of their MHC haplotype: they might have a dominant role for Th sensitization. Figure 4 indicates the putative transmembrane topology of the AChR sequence regions recognized by CD4+ T-cells.

The T-cell epitope repertoire identified so far is certainly not an exhaustive representation of the actual repertoire in the MG patient population. On the other hand, the T-cell repertoire presently identified suffices to conclude that while the antibody response in MG is focused on the MIR (Lindstrom et al., 1988; Schönbeck et al., 1990), the anti-AChR CD4+ T-cells recognize a large number of epitopes. Even the relatively immunodominant epitopes recognized by most MG patients are numerous. This clearly indicates that during the fully developed anti-AChR response, the AChR itself is the target of the CD4+ T-cells. However, it does not exclude the possibility that the anti-AChR response may be triggered by molecular mimicry between an individual AChR epitope and some other microbial structure (Oldstone, 1987). Once the tolerance is broken

Table 7
Human AChR Sequence Regions Known to Form T-Cell Epitopes

Region α1-38	Region α48-71	Region α85-143	Region α146-181	Region α182-212	Region α257-269	Region α293-308	Region α304-337	Region α351-368	Region α387-405	Region α403-437
α1-34[a]	α48-673[c,d]	α85-142[e]	α146-162[b]	α182-198[b]	α257-269[g,h]	α293-308[c]	α304-322[c,d]	α351-368[h]	α387-405[c]	α403-421[c]
α12-27[b]	α56-71[b]	α89-104[b]	α151-168[c]	α191-207[c]			α310-327[h]			α419-437[c,d]
α19-34[c]		α99-116[f]	α158-174[b]	α194-210[b]			α320-337[c,d]			
α23-38[b]		α101-120[c,d]	α169-181[h]	α195-212[g,h]						
		α111-126[b]								
		α118-137[c]								
		α125-143[g]								

Region δ1-20	Region δ61-80	Region δ91-185	Region δ196-290	Region δ346-362	Region δ363-392	Region δ446-496
δ1-20[i,j]	δ61-80[i,j]	δ91-110[i]	δ196-215[i,j]	δ346-362[i]	δ363-386[i]	δ446-465[i]
		δ106-125[i]	δ213-230[i]		δ373-392[i]	δ461-480[i,j]
		δ121-140[i,j]	δ226-245[i]			δ476-496[i]
		δ136-155[i]	δ241-260[i,j]			
		δ151-170[i]	δ256-275[i]			
		δ166-185[i]	δ271-290[i,j]			

Region γ15-49	Region γ60-124	Region γ135-154	Region γ180-202	Region γ203-400	Region γ411-430	Region γ470-495
γ15-34[i]	γ60-79[i,k]	γ135-154[i,k]	γ180-202[i]	γ203-222[i]	γ411-430[i,k]	γ470-489[k]
γ30-49[i,k]	γ75-94[i,k]			γ218-237[i]		γ472-495[i]
	γ90-109[i]			γ233-252[i]		
	γ105-124[i]			γ248-267[i]		
				γ263-273[i]		
				γ269-288[i]		
				γ284-303[i]		
				γ297-312[i,k]		
				γ306-325[i]		
				γ321-340[i,k]		
				γ336-355[k]		
				γ351-370[i]		
				γ366-385[i]		
				γ381-400[i]		

Region β16-50	Region β76-95	Region β181-200	Region β271-290	Region β316-350	Region β361-425	Region β436-455
β16-35[i]	β76-95[i]	β181-200[i]	β271-290[i]	β316-335[i]	β361-380[i]	β436-455[i]
β31-50[i]				β331-350[i]	β376-395[i]	
					β391-410[i]	
					β406-425[i]	

[a]Hohlfeld, 1988. [b]Oshima, 1990. [c]Manfredi, 1992. [d]Protti, 1990. [e]Melms, 1988. [f]Sommer, 1991. [g]Harcourt, 1988. [h]Brocke, 1989. [i]Manfredi, 1993. [j]Protti, 1991. [k]Protti, 1992. [l]Moiola, 1994a.

for a single epitope, an autoimmune response can extend to other Th epitopes within an autoantigen (Lehmann et al., 1992).

C. Structure of Immunodominant Th Epitopes on Muscle AChR: Studies with Single-Residue-Substituted Peptides

Although the Th cells against the AChR in MG recognize many epitopes, a few 20-residue sequence regions are the most frequent target of autoimmune Th cells. This finding could be exploited for development of epitope-specific immunosuppressive procedures, as was done for experimental autoimmune encephalomyelitis (EAE). Four immunodominant regions were identified on the α (Protti et al., 1990b)-, three on the β (Moiola et al., 1994a)-, and two on the γ-subunit (Protti et al., 1992). Because those immunodominant sequence regions are 20 resi-

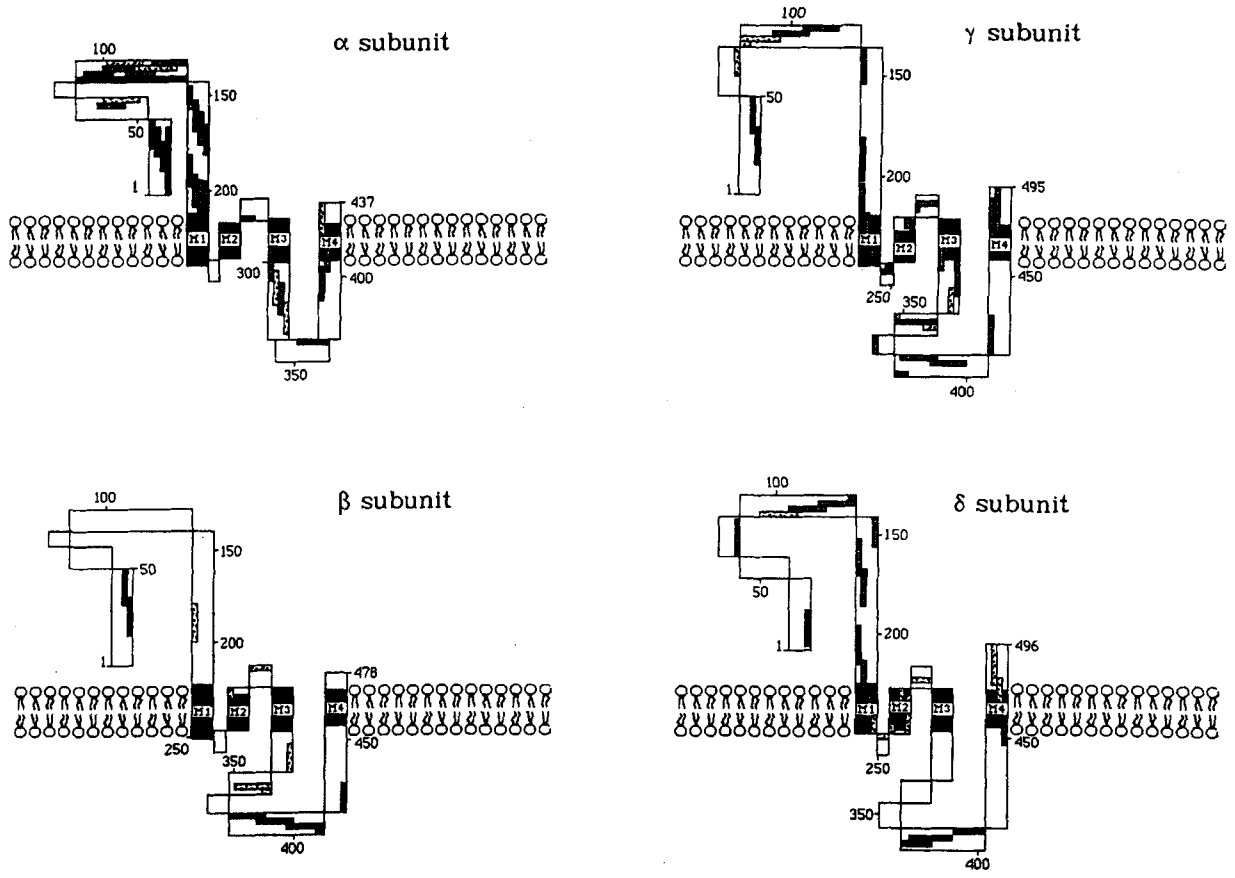

FIGURE 4. Schematic representation of the transmembrane folding of the AChR subunits and location of sequence regions identified as forming epitopes for anti-AChR T cell. The epitopes forming sequence regions are indicated as gray segments, while white dotted segments indicate "immunodominant" peptides recognized by patients of different class II haplotype. The four putative transmembrane regions (M1-M4) are indicated in black. The sequence segment aminoterminal to the M1 region (residues 1 to ~220) is believed to form an extracellular domain; the segment between M3 and M4 forms a cytoplasmic domain. The transmembrane topology of the carboxyl terminal sequence segment following M4 is still under debate but is probably extracellular (see text). (From Protti, M. P., Manfredi, A. A., Horton, R. M., Bellone, M., and Conti-Tronconi, B. M. 1993. *Immunol. Today* **14**:363–368. With permission.)

dues long, and class II-restricted epitopes are 9 to 15 residues (Nicholas et al., 1989; Michalek et al., 1989), each immunodominant synthetic sequence could accommodate several nested epitopes, as described for immunodominant regions of myelin basic protein (Sakai et al., 1988).

The presence of short sequence regions immunodominant for Th sensitization is not unique to the anti-AChR autoimmune response, as it occurs in the human $CD4^+$ T-cell response to foreign protein antigens (Panina-Bordignon et al., 1989; O'Sullivan et al., 1991). This may occur because a variety of peptides contain a consensus sequence compatible with binding to different DR molecules because of the promiscuous nature of the binding of human DR molecules to peptide epitopes: most or all human DR molecules bind a wide spectrum of peptide sequences, and about 25% of the peptide sequences tested bound to any DR molecule (O'Sullivan et al., 1990). It is possible that for any protein antigen, the sequence regions most easily excised during proteolytic processing (e.g., regions largely exposed on the antigen surface, which could be "stripped" with minimal denaturation of the protein) will occupy the

available DR molecules, outcompete antigen sequences processed with slower kinetics, and thus dominate the Th response.

A study employing single-residue-substituted analogs of sequence regions of the human muscle α- and γ-subunits immunodominant for Th sensitization in MG patients indicated that CD4+ T-cells from different patients recognize the same 10- to 12-residue sequence segments (Moiola et al., 1994b). That study also found that in the same patient, the CD4+ T cells recognizing a given immunodominant region are polyclonal and recognize different, overlapping epitopes (Moiola et al., 1994b).

D. Anti-AChR Th Cells Exist in Healthy Subjects and in Experimental Animals

Th cells against autoantigens have been demonstrated in normal subjects (Kartik et al., 1990; Pette et al., 1990; Sommer et al., 1991). Peripheral blood lymphocytes from normal subjects may respond to synthetic sequence segments of the AChR α-subunit (Harcourt et al., 1988), although they do not respond to the complete AChR molecule (Protti et al., 1990b; Conti-Tronconi et al., 1979). AChR-specific T-cell clones were obtained from healthy subjects using a biosynthetic fragment of mouse AChR α-subunit, but much less frequently than from MG patients (Zhang et al., 1990). Propagation of specific Th cell lines from healthy controls using pools of synthetic peptides corresponding to the complete sequence of human AChR subunits was seldom successful (Protti et al., 1990a, b, 1991, 1991a, 1992), although CD4+ lines specific for the human muscle AChR γ- and δ-subunits have been propagated for a short time from normal subjects (Protti et al., 1991, 1991a, 1992). Among the γ-subunit-specific lines, one responded detectably to a pool of synthetic peptides corresponding to the complete γ-subunit sequence, but not any individual peptide, suggesting that the CD4+ T-cells interacted with γ-subunit epitopes with too low affinity for any one peptide epitope to be clearly recognized *in vitro*. Other γ-specific lines recognized some individual peptides, but could not be propagated for longer than 4 to 5 weeks, consistent with inefficient recognition of their epitopes (Protti et al., 1992). Th cells recognizing AChR epitopes with high affinity, which can be propagated *in vitro* for long periods of time by stimulation with AChR antigens, seem to be uniquely characteristic of MG patients.

The results summarized above clearly indicate that potential anti-AChR autoimmune Th cells exist in the healthy population and that, as Barkas and co-workers pointed out, "immunoregulatory imbalance rather than the presence of autoreactive T cell clones is responsible for the pathogenesis of MG" (Zhang et al., 1990). Autoreactive T-cells have been demonstrated in many experimental systems where the host does not normally develop autoimmune diseases (Gammon and Sercaz, 1990), including CD4+ cells able to recognize autologous muscle AChR in mice (Bellone et al., 1991, 1993). Balb/c mice develop EAMG after injection of rat AChR, which is presumably very similar, if not identical, to the autologous murine AChR (Granato et al., 1986), and this mouse strain has CD4+ T-cells that recognize the murine sequence region α304-322 (Bellone et al., 1991). The presence of CD4+ cells reactive to self-antigens does not indicate a failure of tolerance, because the many autoreactive cells that are deleted from the T-cell repertoire during maturation within the thymus are probably those with optimal affinity for the self-antigen/MHC complex (reviewed in Gammon and Sercarz, 1990). T-cells with low-affinity antigen receptors may escape clonal deletion (Gammon and Sercarz, 1989, 1990), but because of the similarity between the mechanism of thymic clonal deletion and peripheral T-cell activation — which both require optimal formation of a ternary complex between the T-cell antigen receptor (TCR), the MHC-restricting element, and the T-epitope — the autoreactive T-cells may never be activated at the periphery because of the low affinity of their TCRs (Gammon and Sercarz, 1989, 1990). Potentially autoreactive T-cells could be activated under special conditions and cause autoimmune responses.

XIII. THE ORIGINAL AUTOANTIGEN IN MG: WHICH AND WHENCE?

It is not known whether the anti-AChR response in MG is initiated by muscle AChR, an

AChR-like protein expressed in another tissue, or a cross-reactive protein. The thymus has been proposed as the organ where the original anti-AChR sensitization takes place. In myasthenic patients, it is frequently hypertrophic, may have a thymoma (Engel, 1987; Levinson et al., 1987; Lindstrom et al., 1988; Schönbeck et al., 1990), and may contain anti-AChR T- and B-cells (Schönbeck et al., 1990). Thymectomy is commonly done in MG patients and is beneficial for their symptoms. Transplantation of thymus fragments from MG patients into SCID mice results in the appearance of human anti-AChR antibodies that bind at the mouse neuromuscular junction and cause a reduced content of muscle AChR (Schönbeck et al., 1992). Therefore, the thymus from MG patients contains all the functional components for development of a pathogenic anti-AChR response.

Different AChR-like proteins have been described in the thymus. One is expressed in thymomas and is not a true AChR because, although it binds certain anti-AChR antibodies, it does not bind α-BTG and contains a single subunit of 153 KDa, approximately three times as large as a true AChR subunit (Schönbeck, et al., 1990). This protein may be involved in the anti-AChR response in MG associated with thymoma but, as it is not expressed in hypertrophic thymus, is unlikely to be involved in the pathogenesis of most cases of MG. Normal thymus and thymomas contain α-BTG-binding proteins that are probably bona fide AChRs, similar or identical to embryonic muscle AChR (see Section III.A.1).

The thymus embryonic AChR may have a role in the pathogenesis of MG, explaining the dilemma that myasthenic patients produce antibodies that uniquely recognize embryonic muscle AChR (Weinberg and Hall, 1979; Vincent et al., 1987). Indeed, it has been proposed that anti-AChR antibodies in MG recognize epitopes either unique to embryonic AChR or common to both the embryonic and the adult form, never epitopes unique to adult AChR (Weinberg and Hall, 1979; Vincent et al., 1987). Most MG patients have Th cells specific for the embryonic AChR, which recognize several epitopes on the γ-subunit (Protti et al., 1991a, 1992), indicating that a true sensitization to embryonic AChR has taken place.

That the anti-AChR response may be initiated by embryonic AChR is supported by the finding, discussed in Section III.A.1, that adult extrinsic ocular muscles — which are preferentially and sometimes uniquely affected by myasthenic symptoms (reviewed in Kaminski et al., 1990) — express both ε- and γ-subunits (Horton et al., 1993).

Other muscle groups might also express embryonic AChR in addition to, or instead of, the adult form. This is suggested by preliminary studies on the expression of γ- and ε-subunits in different adult muscles (Kaminski et al., 1991). Furthermore, isoforms of the α-and β-subunits exist (Claudio, 1989; Goldman and Tanai, 1989; Beeson et al., 1990) (see Section III.A.1). The presence of different AChR isoforms might explain the differential susceptibility of muscle groups to MG symptoms, and special forms of MG where unusual groups of muscle are preferentially or uniquely affected, such as the chronic limb-girdle MG (Oh and Kuruoglu, 1992) or the forms starting with respiratory failure due to involvement of the diaphragm (Nagappan and Klechtko, 1992).

AChR isoforms other than embryonic muscle AChR may exist in the thymus, which receives a cholinergic innervation, as mRNA encoding the neuronal AChR α3 subunit has been detected by PCR (Mihovilovic and Roses, 1993). The impaired cognitive function occasionally described in myasthenia gravis patients could be related to antibodies active against neuronal AChRs (Iwasaki et al., 1990).

XIV. SUMMARY AND CONCLUSIONS

The AChR is the best characterized member of a large family of structurally related ligand-gated ion channel proteins. They have likely evolved from an ancestor ligand-gated ion channel that probably existed as a homomeric complex. Duplication of the gene encoding such an ancestor and its subsequent divergence yielded different subunits comprising a single receptor complex, then various receptor subtypes, and ultimately receptors that bind different ligands.

The AChR subunits were highly conserved through the course of evolution. For instance, corresponding subunits of *Torpedo* and mamma-

lian muscle AChRs share ~60 to 70% amino acid sequence identity, the different subunits forming muscle AChRs (α1, β1, γ, ε, δ) share ~31 to 49% identity, and the neuronal AChR subunits sequenced to date (α2 to α8 and β2 to β4) share ~37 to 68% sequence identity. The subunits of the other members of the AChR family are less conserved. For example, the glycine and $GABA_A$ receptors share only 22 to 34% amino acid sequence identity with the muscle AChR subunits, and more distant relatives of the AChR superfamily — the NMDA and non-NMDA receptors — share <20% sequence identity with the AChR subunits.

Despite the different degrees of sequence divergence among the different members of this family of proteins, several fundamental structural features — such as their pentameric structure formed by homologous or identical subunits similarly arranged around the central ion channel and the transmembrane folding of their subunits — have been preserved in all members of the AChR family. The AChRs therefore are an excellent system to study the structural features required for ion channel gating.

Due to the ease with which the AChR can be purified, especially from fish electric organ, and as a result of almost 20 years of intensive investigations, several structural features of the AChRs are known, even in the absence of high-resolution crystallographic data. These include the overall tridimensional shape of the AChR molecule, the transmembrane folding of at least some regions of its constituent subunits, the identification of sequence regions which contribute to the lining of the ion channel and elucidation of their likely secondary structure, and the identification of the subunits, sequence regions, and individual residues that contribute to the formation of cholinergic binding sites.

MG is a relatively uncommon autoimmune disease, but, because its symptoms are primarily or exclusively because of an autoimmune response against the muscle AChR, which is so well characterized, it is an excellent system to investigate the molecular interactions occurring in a human autoimmune response. Therefore, MG has been, and still is, the target of intensive investigations. A detailed picture at the molecular level of the interactions of this autoantigen with the key elements involved in the autoimmune response, such as the anti-AChR antibodies and the specific Th cells, is now emerging. The structure of epitopes recognized on the human muscle AChR by the autoantibodies and by $CD4^+$ Th cells of myasthenic patients has been, or is being, elucidated. These studies will ultimately result in a better understanding of the pathogenetic mechanisms of an autoimmune response and, hopefully, in the design of antigen-specific immunosuppressive treatments.

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