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Characterization of Binding Sites and Agonist-Induced Desensitization of the Nicotinic Acetylcholine Receptor in *Torpedo* Electric Organ

By

Liren Cao 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirement for the degree of Master of Science

Division of Neuroscience

Edmonton, Alberta

spring, 1999

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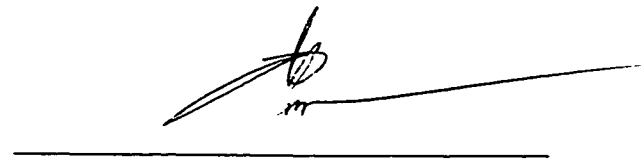

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Characterization of Binding Sites and Agonist-Induced Desensitization of the Nicotinic Acetylcholine Receptor in *Torpedo* Electric Organ** submitted by Liren Cao in partial fulfilment of the requirement for the degree of Master of Science

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Abstract

Competition binding with radiolabelled acetylcholine or suberyldicholine in *Torpedo* nicotinic acetylcholine receptors using dicarboxylic acid bischoline esters gave three groups of K_i values. Short chain esters had values of 100-200 nM; intermediate chain compound K_i values were in the range 10-30 nM while suberyldicholine and longer compounds yielded values of 2-5 nM. In fluorescence titration experiments, short chain esters had K_d values around 1.2 μM and glutaryldicholine and longer esters returned values of about 0.3 μM. These data are interpreted to indicate the presence of subsidiary binding sites located at a distance of 13.9Å from the primary binding sites.

Single channel currents were examined by patch techniques using receptors reconstituted in giant liposomes. Both agonists induced desensitization. Two conductances were seen with both carbachol and arecolone, but the latter induced predominantly the subconductance state. This supported the hypothesis that successive binding states are involved in receptor activation.

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ABBREVIATIONS

Ach	acetylcholine
AchE	acetylcholinesterase
α-BuTx	α-bungarotoxin
AddCh	adipyldicholine
Asp	aspartic acid
AzdCh	azelyldicholine
BSA	bovine serum albumin
BrAch	bromoacetylcholine
Carb	carbachol
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio-]-1-propane sulfonate
α-Ntx	α-cobratoxin
Ci	curie
Cys	cysteine
DAPA	bis(3-azidopyridium)-1,10-decane perchlorate
DedCh	decyldicholine
α-Dtx	α-dendrotoxin
DNPP	diethyl-*p*-nitrophenylphosphate
DDF	p(*N, N*)-dimethyl-aminobenzene diazonium fluoroborate
DMT	dimethyl-*d*-tubocurarine
DTT	dithiothreitol
DodCh	dodecyldicholine
d-TC	*d*-tubocurarine
EDTA	ethylenedinitrilo tetraacetic acid
GABA	γ-aminobutyric acid
GΩ	gigaohm
Glu	glutamic acid
GldCh	glutaryldicholine
HEPES	*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid

5-HT	5-hydroxytryptamine
HTX	histrionicotoxin
K_i	inhibitory dissociation constant
IANBD	4-[*N*-[(2-iodoacetoxy)ethyl]-*N*-methylamino]-7-nitrobenz-2-oxa-1,3-diazole
IAS	5-(iodoacetamido)salicylic acid
κ-BuTx	κ-bungarotoxin
K_d	dissociation constant
Leu	leucine
MBTA	4-(*N*-maleimido)-benzyltrimethylammonium iodide
MadCh	malonyldicholine
B_{max}	maximum receptor binding sites
MΩ	megaohm
MeI	methiodide
μM	micromolar
mM	millimolar
mV	millivolt
nAchR	nicotinic acetylcholine receptor
nM	nanomolar
NBD	7-nitrobenz-2-oxa-1,3-diazole
NCI	noncompetitive inhibitor
OxdCh	oxalyldicholine
PCP	phencyclidine
PMSF	phenylmethylsulfonyl fluoride
pA	picoampere
pS	picosiemen
PidCh	pimelyldicholine
Pro	proline
rpm	rotations per minute
γ_s	short γ subunit
SdCh	suberyldicholine

SudCh	succinyldicholine
τ	time constant
Tris	Tris(hydroxymethyl)aminomethane
Trp	tryptophan
Tyr	tyrosine
v/v	volume per volume
w/v	weight per volume

Chapter 1

Characterization of Binding Sites of Nicotinic Acetylcholine Receptor

1. Introduction

1. 1. Introduction and Background

Nicotinic acetylcholine receptors (nAchR) are the best understood members of a large superfamily of ligand-gated ion channel proteins. Receptors within this family, despite their different ligand specificities and ion gating preferences, share a common structure and have likely evolved from a common ancestral gene. The members of the superfamily include the neuronal and muscle nicotinic receptors (Deneris *et al.*, 1989; Galzi *et al.*, 1991a; Sargent, 1993; Karlin & Akabas, 1995), glycine (Grenningloh *et al.*, 1987; Betz, 1990), γ-aminobutyric acid ($GABA_A$) (Schofield *et al.*, 1987; Dunn *et al.*, 1994) and 5-hydroxytryptamine ($5\text{-}HT_3$) receptors (Maricq *et al.*, 1991). Each receptor contains a binding site or sites for agonists and competitive antagonists and an integral ion channel. Activation of the receptor upon agonist binding opens the channel and allows ions to flow into or out of the cell. The net ion movement across the membrane constitutes a current that generates a rapid electric signal being either depolarizing (excitatory) or hyperpolarizing (inhibitory) depending on the ion selectivity of the receptor ion channel. Therefore, these receptors mediate fast synaptic transmission in synapses throughout the central and peripheral nervous systems.

The peripheral nAchR was the first receptor to be purified and sequenced, and it acquired its role as the prototype of this receptor family primarily because its presence in large quantities in *Torpedo* electric organ facilitated its biochemical characterization. In addition, the contemporaneous development of snake toxin-dependent affinity

chromatography enabled the receptor to be purified and characterized (Stroud *et al.*, 1990; Dunn, 1993; Karlin, 1993; Lena and Changeux, 1993; Conti-Tronconi *et al.*, 1994; Karlin and Akabas, 1995; Hucho *et al.*, 1996; McLane *et al.*, 1996; Arias, 1997).

Peripheral nAchRs are mainly located at the endplate region of the neuromuscular junction and in the *Torpedo* electric organs. Immunological studies and comparison of the primary sequences have shown that the nAcChR subunits from electric fish are highly homologous to those from the mammalian neuromuscular junction (Conti-Tronconi and Raftery, 1982). After synthesis, acetylcholine (Ach) molecules are stored in vesicles in the nerve terminal. Upon arrival of an action potential, these molecules are released in a quantal fashion, diffuse across the synaptic cleft and interact with the receptors embedded in the postsynaptic membrane. This interaction induces a rapid conformational change of the receptor to open the ion channel, with a delay of only 0.3 msec from the time of neurotransmitter release, allowing Na^+ and K^+ ions to permeate down their electrochemical gradient at a rate of approximately 10^7 ions/sec/channel (Dwyer *et al.*, 1980; Hille, 1992). This sodium influx depolarizes the cell membrane, and consequently triggers muscle contraction. The concentration of Ach in the synaptic cleft increases rapidly to millimolar levels, and this concentration is maintained transiently before decreasing rapidly to an undetectable level because of its rapid hydrolysis by acetylcholinesterase (AchE) which is located on the basal lamina (Katz and Miledi, 1977). This rapid change of Ach concentration results in a transient channel open state with a lifetime of less than one millisecond at the frog neuromuscular junction (Katz and Miledi, 1971; Magleby and Stevens, 1972), after which the channel closes and synaptic transmission is terminated. However, if continuously

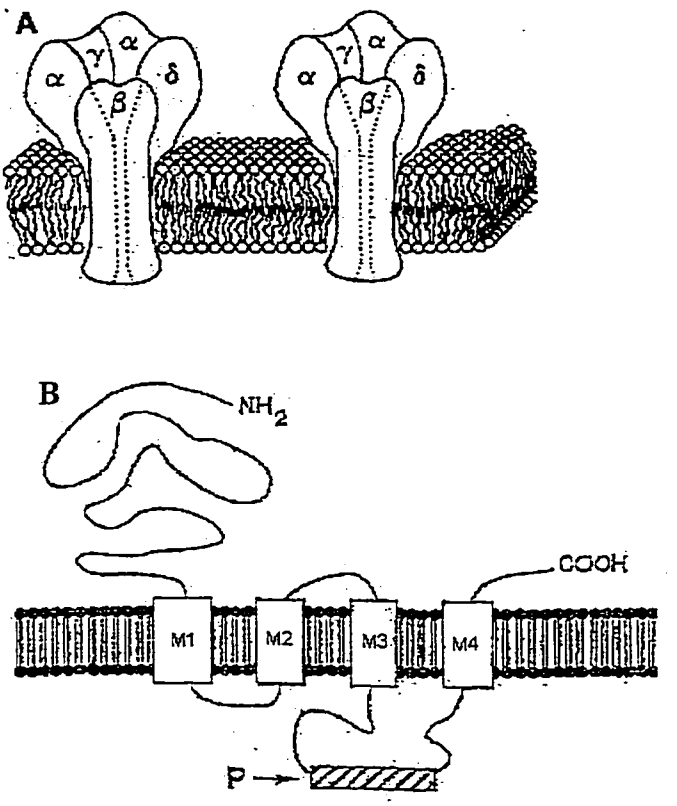


Figure 1. Schematic model of the topology of the nicotinic acetylcholine receptor. (A). Pentameric subunit structure viewed through the plane of the membrane. (B). Membrane topology, viewed through the plane of the membrane of an individual subunit containing extracellular N- and C-terminals and four transmembrane domains M1-M4. N-terminal domains of the two α-subunits and adjacent subunits are believed to possess the high-affinity binding sites for cholinergic agonists and competitive antagonists. P indicates the putative phosphorylation sites for the various protein kinases.

exposed to agonists, the receptor undergoes further conformational changes leading to a nonresponsive desensitized state in which the channel is closed and the affinity for agonists is dramatically increased (Ochoa *et al.*, 1989). The nAchR must therefore exist in at least three distinct conformations, which have different affinities for Ach: a resting but activatable state with no Ach bound and the channel mainly closed; an activated state with Ach bound (K_d~100 μM) and the channel opened; and a desensitized state with Ach tightly bound (K_d~10 nM) and the channel closed (Dunn, 1993; Devillers-Triery *et al.*, 1993)

1. 2. Transmembrane Topology of the Receptor Protein:

The nAcChR, purified from *Torpedo* electric organ, is a pentamer. It consists of four homologous subunits assembled in a stoichiometry of $\alpha_2\beta\gamma\delta$ around a central cation channel (Figure 1. Raftery *et al.*, 1980; Noda *et al.*, 1982,1983a,b). Each subunit contains a long N-terminal and a short C-terminal domain, both of which are believed to orient extracellularly. The hydrophilic N-terminal domain has more than 200 amino acids followed by four putative homologous hydrophobic segments, referred to as M1-M4, which are likely to form the membrane-spanning regions (Figure 1; Dunn, 1993; Karlin, 1993; Karlin & Akabas, 1995). Between M3 and M4, there is a long (109-142 amino acids) intracellular loop which contains all of the receptor's putative phosphorylation sites (Huganir & Greengard ,1987). The results of mutagenesis deletion experiments suggest that the putative intracellular domains do not play a significant role in the ligand-gated functioning of the receptor (Mishina *et al.*, 1985) However, the potential phosphorylation sites can be phosphorylated by serine/threonine protein kinases and tyrosine kinases and this may be involved in the enhancement of receptor desensitization (Ochoa *et al.*, 1989; Paradiso and Brehm, 1998).

The nAchR from *Torpedo* occurs in two forms: as a monomer (9.0 S) and as a dimer (13.7S) (Raftery *et al.*, 1972). The dimeric form predominates in the postsynaptic membrane and is stabilized by cysteine residues which form a disulfide bond between the δ subunits of two neighboring receptor monomers (Suarez-Isla & Hucho, 1977; Hucho *et al.*, 1978; Dunn *et al.*,1986). The importance of this dimerization remains unclear, since it has not been found in other members of this ligand-gated receptor superfamily and no differences in ligand binding and channel properties of the monomers and dimers have been observed (Anholt *et al.*, 1980; Fels *et al.*, 1982).

A number of studies strongly support the idea that the two α subunits are not adjacent neighbors in the pentamer and that the γ subunit separates the two α subunits (Karlin *et al.*, 1983; Blount and Merlie, 1989; Pedersen and Cohen, 1990; Sine and Claudio, 1991; Kreienkamp *et al.*, 1995; Machold *et al.*, 1995). Therefore, the putative arrangement of nAchR subunit may be α-γ-α-δ-β, as viewed from synaptic cleft (Hucho *et al.*, 1996). However, there is also contradictory evidence suggesting the subunit arrangement to be α-β-α-γ-δ (Kubalek *et al.*, 1987; Chatrenet *et al.*, 1990).

1. 3. Three-Dimensional Structure of *Torpedo* nAchR

The approximate shape of nAchR has been studied by electron microscopy and low angle x-ray diffraction of membranes enriched in the receptor complex (Brisson and Unwin, 1985; McCarthy *et al.*, 1986). The nAchR is a cylinder-like molecule and a view from the top shows that it is an 80 Å diameter rosette with a central pit approximately 25 Å in diameter. Five peaks of electron density pseudo-symmetrically arranged around an axis virtually perpendicular to the plane of the membrane suggest that the five subunits form a

pentagonal complex. In side view, the subunits appear to be 110 Å long in the natural state and 130 Å after alkali purification, due to disordering of the protein domains (McLane *et al.*, 1996). Each subunit extends about 55-65 Å from the extracellular surface and 15 Å from the cytoplasmic surface of the membrane. Thus only about 35% of the protein is immersed in the membrane and about 50% is extracellular (Figure 1; Mitra *et al.*, 1989; Unwin, 1996; Hucho *et al.*, 1996).

Sophisticated techniques of analytical electron microscopy allowed Unwin (1993) to propose a three-dimensional structure for the receptor. Each subunit contains three rods assumed to be α helices, which are situated about 30 Å above the membrane bilayer and may be involved in forming the binding pocket for Ach. A fluorescence study supported the idea that Ach binding sites are located approximately 25 Å below the extracellular apex of the nAchR (Valenzuela *et al.*, 1994). The conformations of the α-subunits were further examined more recently by determining the projection structure of the receptor to a resolution of 7.5 Å (Unwin, 1996). It was concluded that each α-subunit consists of a cavity encircled by three α-helical rods. This cavity was speculated to be the Ach-binding pocket, and its affinity for Ach may be determined primarily by the orientations of these rods.

1. 4. The Ligand-Binding Sites

1. 4. 1. Cholinergic Agonists and Competitive Antagonists

Cholinergic agonists are ligands that bind to and thereby activate the receptor by opening the channel. There are at least two possible kinetic mechanisms for receptor activation by agonist: conformational induction, in which an agonist initially binds to the resting state of the receptor and subsequently induces conformational changes leading to the

active state; and conformational selection, in which the unliganded receptor transiently shifts between the active and inactive states and the agonist then selects the active conformation by binding to the receptor and locking it in this state (Kenakin, 1995,1996). There is argument about which mechanism is the predominant one for agonism, however, there is evidence to suggest that receptor activation more likely proceeds by conformational induction rather than conformational selection (Bruns, 1996). Cholinergic antagonists, on the other hand, are compounds that compete for the same binding sites as agonists and thus inhibit the action of agonists. In the cases of competitive antagonists, their occupancy can be overcome by increasing the concentration of agonists. They bind with similar affinity to resting and desensitized states of nAchR while agonists bind with 10^5-fold greater affinity to the desensitized state than the resting state (Neubig *et al.*,1982; Dunn, 1993; Devillers-Triery *et al.*, 1993).

In addition to the conformational changes of the receptor due to binding of cholinergic ligand, there are also conformational changes of ligand structure resulting from the binding. Ach is a relatively flexible compound that could adjust its three-dimensional structure accommodating to the geometry of the binding sites. The optimal geometric requirements for Ach binding are a positively-charged quaternary ammonium group and a hydrogen bond acceptor (e.g. a carbonyl oxygen) separated by 5.9 Å (Beers and Reich, 1970). When binding to the receptor, this distance shortens to 3.3 Å with the acetyl group moving closer to the choline moiety (Behling *et al.*,1988). By using two-dimensional NMR analysis, Behling *et al.* (1988) suggested that the conformations of Ach in the free and bound states are different. In the free state, the molecule is in the most stable, lowest free energy

gauche conformation. When bound to the desensitized receptor, Ach is in a high energy eclipse conformation. This bound-state conformation places the two electronegative oxygens on the same side of Ach molecule, and the hydrophobic acetyl methyl and choline methylene groups form a hydrophobic surface over the rest of the molecule (Figure 2). A conformational re-arrangement was also observed upon association of the competitive antagonist, d-tubocurarine (d-TC), with the receptor (Fraenkel *et al.*, 1994). Free d-TC is a relatively rigid ligand with two quaternary nitrogens separated by 10.8 Å to maximize the repulsion between them. When bound to the receptor, d-TC is bent with this distance shortening to 6 Å.

1. 4. 2. Binding Sites for Agonists and Competitive Antagonists

The equilibrium binding of cholinergic ligands to the purified and membrane-bound receptors has been extensively studied (Conti-Tronconi and Raftery, 1982; Raftery *et al.*, 1983; Stroud *et al.*,1990; Galzi *et al.*, 1991a). It is well established that each nAchR has two high-affinity binding sites for agonists and competitive antagonists, including peptide α-neurotoxins, in the desensitized state of the receptor (Conti-Tronconi and Raftery, 1982).

Early affinity labeling experiments showed that these high-affinity sites are located, at least in part, on the extracellular domain of the two α subunits. Following reduction of a reactive disulfide bond formed by adjacent cysteines α-192/α-193, these cysteine residues could be labeled by the affinity alkylating agonist, [^{3}H]bromoacetylcholine (BrAch), or antagonist, [^{3}H]MBTA [4-(N-maleimido)-benzyltrimethylammonium iodide] (Moore and Raftery, 1979; Kao *et al.*, 1984; Kao and Karlin, 1986). Thus the two cysteine residues are speculated to be located close to the high affinity binding sites.

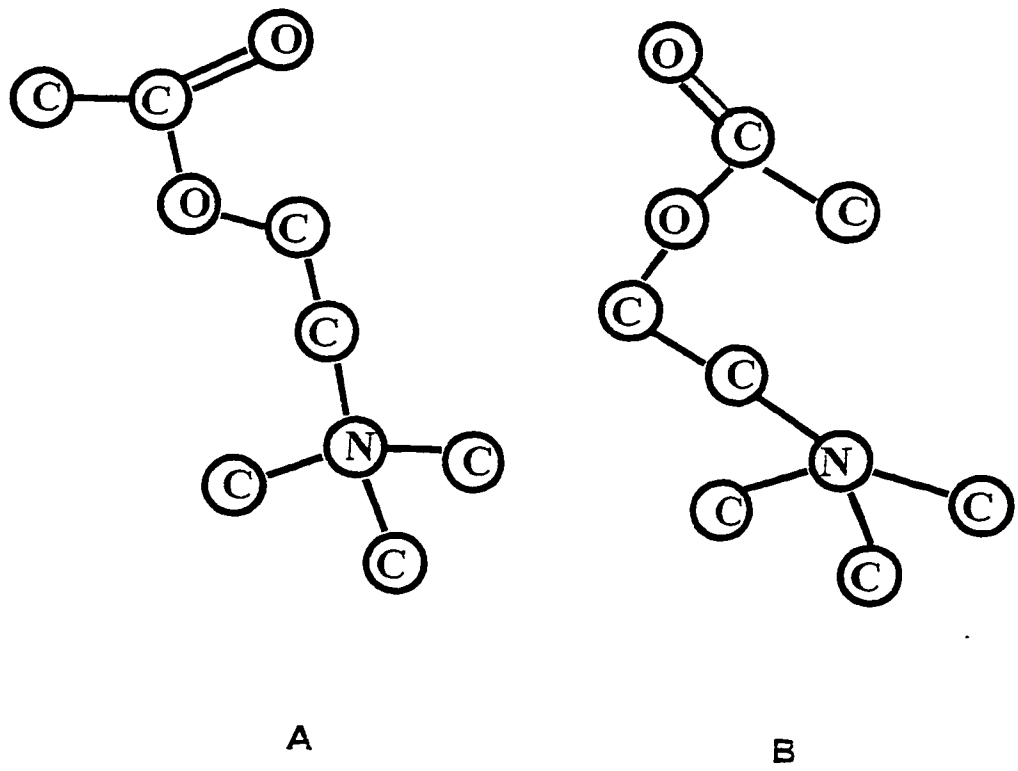


Figure 2. Conformations of acetylcholine in its free (A) and bound (B) states. In free state, acetylcholine in the most stable gauche conformation with the two oxygens in the opposite sides of the molecule. The distance between the quaternary nitrogen and the carbonyl oxygen is 5.9 Å. When bound to the receptor, this distance will be shortened to 3.3 Å. The conformation of acetylcholine molecule changes to a less stable eclipse state with the two oxygens residing at the same side of Ach molecule.

From the results of photoaffinity labeling by using p(N,N)-dimethyl-aminobenzene diazonium fluoroborate (DDF), Changeux and his colleagues proposed a *three-loop* model of the agonist and competitive antagonist binding sites in the N-terminal domain of the α subunits in that the labeled amino acids belong to three different regions (Dennis *et al.*, 1986,1988; Galzi *et al.*, 1990, 1991a; Changeux *et al.*, 1992). This antagonist probe DDF could be efficiently photoincorporated into the α-subunit, with mainly specific photolabeling of Tyr-190 and Cys 192/193, and to a lesser extent Trp-149 and Tyr-93. The labeling pattern with DDF was found to be dependent on the functional state of the receptor in which α-Tyr-93 and α-Trp-149 were labeled to a greater extent in the desensitized state (Galzi *et al.*, 1991b). In addition, α-Tyr-190 was observed to be covalently labeled by other competitive antagonists, i.e. lophotoxin (Abramson *et al.*,1989) and d-tubocurarine (d-TC; Pedersen and Cohen, 1990). Moreover, an agonist affinity label [^{3}H]Ach mustard identified α-Tyr-93 (Cohen *et al.*, 1991). A partial agonist [^{3}H]nicotine was observed to label α-Tyr-198 and to a lesser extent α-Cys-192 and Tyr-190 at equilibrium (Middleton and Cohen, 1991). According to this three-loop model, three distinct regions, centered around residues 93, 149-152 and 180-200 of α-subunit, are believed to form part of the binding site of agonists and antagonists. The functional significance of these amino acids was further explored by site-directed mutagenesis and electrophysiological recording using the *Xenopus* oocyte expression system. Various mutations of the four aromatic residues (Tyr-93, Trp-149, Tyr-190 and Tyr-198 of the α-subunit) caused 5-fold to 100-fold reduction of the receptor affinity for Ach (Galzi *et al.*, 1991c; Tomaselli *et al.*, 1991; O'Leary and White, 1992; Aylwin and White, 1994). Also, d-TC affinity was indicated to be significantly reduced when α-Tyr-93

and α-Tyr-190 were substituted by phenylalanines (O'Leary *et al.*,1994). Mutation of the vicinal α-Cys-192/193 to serine exhibited a 10- to 30-fold reduction in binding affinity for the agonist carbachol (Carb). Therefore, the binding site is not a vicinal linear sequence, but rather is formed by several discontinuous sequences within the extracellular regions of the α-subunit. Also, competitive studies using monoclonal antibodies directed at different receptor epitopes, further supported the involvement of these different binding subregions (Watters and Maelicke, 1983; Mihovilovic and Richman, 1987). Four different mAbs blocked both α-BuTx and Carb binding to *Torpedo* AchR but did not affect d-TC binding, thus distinguishing different subsites for these ligands. Another mAb did not affect agonist binding, but inhibited ~50% of both α-BuTx binding and high-affinity d-TC binding. Similarly, cholinergic agonists and neurotoxins but not d-TC and hexamethonium compete with an anti-AchR mAb (Chinchetru *et al.*,1989). From these results, it is likely that the binding sites for agonists and antagonists are distinct but overlapping (McLane *et al.*, 1996).

Those affinity-labeled and photoaffinity-labeled aromatic residues and the adjacent cysteines of α-subunits are conserved at homologous positions in all known muscle and neuronal nAch receptors. They are proposed to form an aromatic gorge or pocket to accommodate the positively charged quaternary ammonium group of Ach (Changeux *et al.*, 1992; Changeux, 1995; Hucho *et al.*, 1996). A similar structure was reported to exist in AchE, in which the trimethylammonium group is bound primarily by aromatic residues in the Ach binding site (Sussman *et al.*, 1991). Recent studies indicate that these aromatic amino acids are critical for Ach binding through cation-π interactions together with electrostatic interactions between the quaternary ammonium cation and the carboxylate

anions (Dougherty and Stauffer, 1990; Dougherty, 1996). The cation-π interaction is suggested to be a stabilizing attraction between the positively-charged quaternary ammonium group and the π electrons of electron-rich aromatic rings of the Tyr and Trp residues in particular. By using a 9 Å long cross-linker and site-directed mutagenesis, Karlin and coworkers demonstrated that negatively charged carboxylate residues of γ Asp-174, δ Asp-180 and δ Glu-189 are at a 9 Å distance from α Cys-192/193 and contribute to the binding of Ach (Czajkowski and Karlin, 1991,1995; Czajkowski *et al.*, 1993; Stauffer and Karlin, 1994). When Ach binds, these negatively charged residues would move toward α cys-192/193 so as to make closer, lower energy consuming contact with agonist in the transitions leading from the resting state to the active and desensitization states (Karlin and Akabas, 1995). Therefore, Ach binding sites contain both acidic and aromatic amino acids. The acidic side chains could provide long-range charge-charge interaction with Ach, while the aromatic side chains could provide short-range cation-π-electron and hydrophobic interactions (Stauffer and Karlin, 1994). On the other hand, the positively-charged quaternary group of Ach plays a key role in receptor binding in which it provides interactions with both π electrons of aromatic residues and acidic residues.

Recent photoaffinity labeling studies with [^{3}H]nicotine (Middleton and Cohen, 1991), d-[^{3}H]TC (Pedersen and Cohen, 1990) and an affinity labeling study using [^{3}H]BrAch (Dunn *et al.*, 1993) have indicated that the ligand binding sites are not associated exclusively with the α subunits, but rather may be located at the α-γ and α-δ subunit interfaces. In muscle AchRs, coexpression of α/γ and/or α/δ subunit pairs was necessary to determine the binding affinity for d-TC, whereas α subunits alone could not (Blount and Merlie, 1989; Gu *et*

al.,1991; Sine and Claudio, 1991). In an early saturation binding study of [^{3}H]d-TC to *Torpedo* membranes, two affinities of 30 nM and 7 μM with equal numbers of binding sites were described (Neubig and Cohen, 1979). Since the two α-subunits are encoded by a single gene (Merlie *et al.*,1983; Klarsfeld *et al.*, 1984), have an identical primary sequence, and are likely to have similar secondary and tertiary structures (Galzi *et al.*, 1991a,b; Changeux *et al.*, 1992), the γ and δ subunits are more likely to account for the non-equivalence of d-TC binding to native nAchR. Photolabeling studies by Pedersen and Cohen (1990) suggested that the α-γ subunit interface constitutes the high-affinity site for d-TC, whereas the low-affinity site resides at the interface between the α- and δ-subunits. Moreover, two other aromatic residues, δ-Trp-57 and γ-Trp-55, were found in the d-TC binding sites using photoinduced cross-linking (Cohen *et al.*, 1992; Chiara and Cohen, 1997). Mutation of γ-Trp-55 and δ-Trp-57 of mouse muscle had moderate effects on the binding of cholinergic agonists (O'Leary *et al.*, 1994). Thus, γ and δ subunits were speculated to contribute a fourth loop to form the two binding sites for agonists and antagonists. In electron microscopic studies, Unwin (1993,1996) proposed that these non-α-subunits are involved in ligand binding only indirectly by shaping the binding pockets and by facilitating the access of ligands to these pockets.

Taken together, the binding sites for cholinergic agonists and competitive antagonists is speculated to be a gorge, tunnel or pocket. They would be situated at the interfaces between the α/γ and α/δ subunits, respectively, approximately 30 Å above the membrane bilayer and also 20 Å away from the channel (Machold *et al.*, 1995). It contains tandem cysteine residues and several aromatic amino acid side chains occurring in at least three

loops within the extracellular domain of the α-subunits with a fourth loop being contributed by the neighboring γ- and δ-subunits. After ligand binding, these discontinuous residues fold together in order to accommodate the bound ligand.

The binding of agonists and competitive antagonists to nAChR exhibit different properties. Neubig and Cohen (1979) demonstrated that some flexible and rigid competitive antagonists, e.g. d-tubocurarine (d-TC) and gallamine, displayed two distinct high and low affinities in their displacement of [^{3}H]Ach, presumed to be due to the differences between the sites at the α-γ and α-δ subunit interfaces. Likewise, these differences between the subunit interfaces were also suggested to account for the widely different affinities for Ach at equilibrium with high-affinity site provided by α-γ subunit interface and low-affinity by α-δ interface (Sine *et al.*,1990; Sine, 1993; Zhang *et al.*, 1995). These authors suggested that one agonist molecule binding at one subunit interface would lead to positive cooperativity to the other site for the second molecule. However, there is also evidence that agonists, e.g. Ach and suberyldicholine (SdCh) showed only one affinity without cooperativity at equilibrium (Dunn, 1993; Dunn and Raftery, 1997a). This indicates that subunit interfaces between α-γ and α-δ may provide similar geometric environment for the bound agonist molecule. Recent thermodynamic studies further suggested that the energetics of the binding of cholinergic agonists and antagonists may differ, in which the binding of agonist is enthalpy-dependent to induce rigid conformational changes of the receptor whereas entropy-controlled antagonist binding leads to flexible conformational changes (Banerjee and Ganguly, 1995, 1996).

In addition to the channel opening induced by agonist binding, electrophysiological

studies have suggested that agonists can bind to an additional site in the open state, leading to inhibition of ion flux, referred to as self-inhibition (Sine and Steinbach, 1984; Ogden and Colquhoun, 1985; Carter and Oswald, 1993). This usually requires sufficiently high agonist concentrations, e.g. several hundred micromolar or even millimolar levels, for the agonist molecule to enter and transiently occlude the ion-channel. In this way, agonists may act as noncompetitive channel inhibitors (NCI) as well as activators.

1. 4. 3. Binding Sites for α-Neurotoxins

α-Neurotoxins constitute a large number of polypeptide cholinergic antagonists that bind specifically and with high affinity to the nAchR. α-BuTx is the most potent inhibitor known so far for the nAchR from the *Torpedo* electroplax. Early studies demonstrated that each native receptor in its membrane-bound state carries two binding sites for α-BuTx with different affinities (Conti-Tronconi *et al.*,1990a), one being of extraordinarily high affinity (10^{-12} to 10^{-10} M) and slow dissociation rate, the other being of much lower affinity (~ 60 nM) and readily reversible. In the absence of γ- and δ-subunits, α-BuTx was reported to bind to isolated α-subunits, albeit with lower affinity (Blount and Merlie, 1988). This evidence suggests that α-BuTx binding sites are located on the α-subunits but not necessarily exclusively. By testing a panel of synthetic peptides, Conti-Tronconi *et al.*(1990b) further defined two sequence segments α-181-200 and α-55-74 contributing to the α-BuTx binding site on the *Torpedo* nAchR. Recent site-directed mutagenesis studies further identified several aromatic amino acid residues involved in α-BuTx binding. It appears that α-Tyr-187, α-Tyr-189, α-Tyr-190 and α-Tyr-198 play a critical role in this interaction, and substitution of these amino acids dramatically decreased the affinity for α-BuTx (McLane *et al.*,1990,

1991a,b, 1994; Barchan *et al.*,1995; Lentz, 1995; Ackermann and Taylor, 1997). The adjacent cysteine pair, α-Cys-192/193, are also intimately involved in forming contacts with α-BuTx, but appear less critical (McLane *et al.*, 1991a,b; Balass *et al.*, 1997). In addition to the α-subunit, γ- and δ-subunits were also suggested to play an important role in toxin recognition (McLane *et al.*,1996; Ackermann and Taylor, 1997). Therefore, α-BuTx binding, like the agonists and antagonists already mentioned, involves multiple but distinct attachments of aromatic amino acids on the α- and non-α-subunits particularly at the α/γ and α/δ interfaces. Even though α-BuTx is very potent in blocking muscle nAchR, it does not have a comparable effect on most neuronal nAchRs, which are either homomeric complexes or heteromeric complexes composed of α- and β- subunits. The brain α-7 isoform can form a homomeric complex that is sensitive to α-BuTx. On the other hand, κ-bungarotoxin (κ-BuTx) is a specific antagonist for most neuronal AchRs which are α-BuTx insensitive (McLane *et al.*,1996). Other neurotoxins, including α-cobratoxin (α-Ntx) (Conti-Tronconi and Raftery, 1986) and lophotoxin (Culver *et al.*,1984) also bind to two sites per receptor molecule. As mentioned above, α-Tyr-190 was identified to be photolabeled by lophotoxin (Abramson *et al.*,1989). In contrast, α-dendrotoxin (α-Dtx) was observed to bind to the membrane-bound *Torpedo* AchR with a stoichiometry of four per receptor molecule (Conti-Tronconi and Raftery, 1986). Moreover, these authors found that when the AchR was saturated with α-BuTx, addition of α-Dtx significantly accelerated the dissociation of bound α-BuTx. This indicated that the occupancy of the additional two sites by α-Dtx influences and reduces the affinity of α-BuTx for its two binding sites. This could be explained as cooperative interactions between the sites.

1. 4. 4. Multiple-Subsite Model

Previous experiments showed that [^{3}H]Ach mustard (Middleton and Cohen, 1991) and [^{3}H]nicotine (Cohen *et al.*, 1991) specifically labeled α-Tyr-93 and α-Tyr-198, respectively, but not both. This implies that the binding site for Ach may be distinct from that for nicotine and other agonists, and formation of these (sub)sites probably involve several peptide loops of the α-subunit. This conclusion was also substantiated by some studies employing various monoclonal antibodies recognizing different epitopes within the cholinergic binding site (Watters and Maelicke, 1983; Mihovilovic and Richman, 1987). More recently, studies of the kinetics of association and dissociation using both radiolabeled agonists and fluorescence techniques have suggested that each high affinity sites consists of two subsites which are mutually exclusive at equilibrium (Dunn and Raftery, 1997a,b). In the dissociation study (Dunn and Raftery, 1997a), micromolar concentrations of unlabeled agonists significantly increased the dissociation rate of bound [^{3}H]AcCh in a concentration-dependent manner even when the [^{3}H]Ach originally saturated all the high-affinity sites. However, the dissociation rate of [^{3}H]suberyldicholine (SdCh) was only marginally affected by various concentrations of unlabeled agonists. To explain this observation, a model (Figure 3) was proposed that suggested the presence of an additional site (or subsite) for Ach with an affinity in the micromolar range. According to this multiple-subsite model, if [^{3}H]Ach, a mono-quaternary ligand, originally occupies site A with high affinity, the binding of an additional Ach molecule to site B with an affinity in the micromolar range drastically reduces the affinity of site A and accelerates the [^{3}H]Ach dissociation rate. At equilibrium, only one Ach molecule binds to either subsite A or B, but not both. In contrast to [^{3}H]Ach,

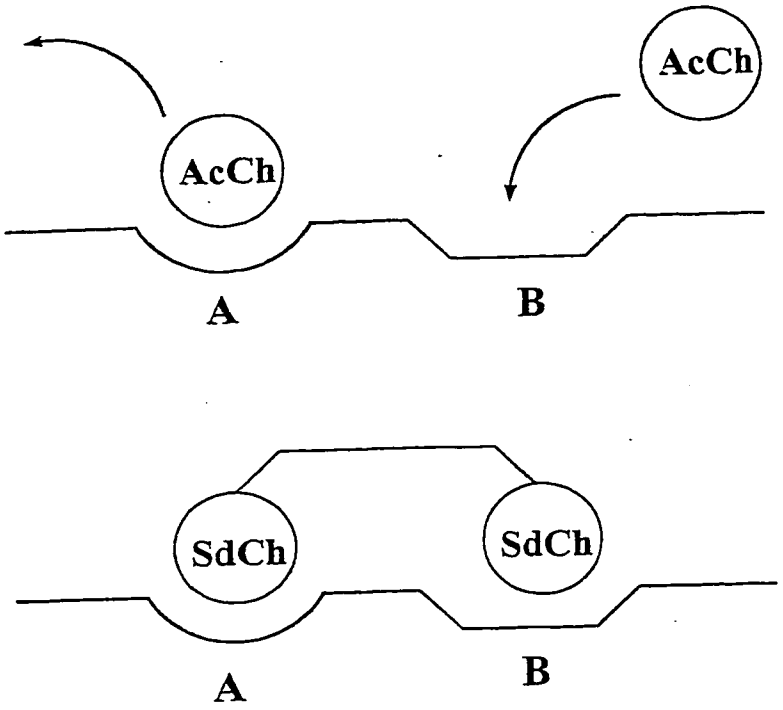


Figure 3. Two-subsite model as recently suggested (Dunn and Raftery 1997a, b). Site A is primary and site B is secondary, both of which are mutually exclusive. At low ligand concentrations, one molecule of monoquaternary ligand can occupy the primary site A. The secondary site B can only be occupied by another molecule when essentially high ligand concentrations are used. This occupancy would dramatically increase dissociation rate of the molecule from the primary site A. At equilibrium, only one site, either A or B, is occupied under the conditions of their experiments. On the other hand, a large bis-quaternary ligand can bridge the two subsites simultaneously as its length places each binding moiety in close proximity to the receptor binding sites providing the distance between the quaternary nitrogens (the inter-onium distance) is sufficiently great.

the dissociation of [^{3}H]SdCh, a large flexible bis-quaternary agonist, was much less affected by the presence of unlabeled ligands. It was therefore, suggested that this bis-quaternary ligand is able either to bridge the subsites physically or at least inhibit site B sterically so as to prevent a second molecule from binding.

Recently, stopped-flow techniques were used to examine the kinetics of binding of the cholinergic agonists, carbachol (Carb) and SdCh, and also a series of bis-choline esters of dicarboxylic acids, to fluorophore-labeled nAchR (Dunn and Raftery, 1997b). It was found that the Carb-induced fluorescence enhancement took place in three distinct phases which have been attributed to conformational changes of the protein. The rates and amplitude of these phases were concentration-dependent and the dominant feature of the kinetics was an obvious slow phase of fluorescence enhancement at low concentration of Carb which was replaced by a much faster phase as higher Carb concentration was applied. Detailed analysis of the kinetics led to the suggestion that at the higher concentrations the second site B could be occupied and this occupancy would induce the receptor to reach the equilibrium more quickly. On the other hand, the kinetics of SdCh displayed a biphasic time course, with both phases becoming slightly faster at higher concentrations. The observations led to the suggestion that SdCh may occupy both subsites simultaneously due to its size and bis-quaternary character and this would prevent second molecule from binding regardless of the various concentrations were used. When the series of bischolines was investigated, it was found that shorter chain dicarboxylic acid esters displayed Carb-like behavior in their kinetics while pimelyldicholine (C7) and longer chain ligands behaved similarly to SdCh (Dunn and Raftery, 1997b). A clear break of their affinities was obtained which proposed

that the longer bischolines after the affinity break may be long enough to span the two subsites and shorter ligands may not.

1. 4. 5. Low-Affinity Binding Sites

In addition to the two high-affinity binding sites, distinct low-affinity binding sites for cholinergic agonists have been identified by monitoring fluorescence changes of 4-[N-(iodoacetoxy)ethyl-N-methyl]amino-7-nitrobenz-2-oxa-1,3-diazole (IANBD)-labeled nAchR induced by agonist binding (Conti-Tronconi *et al.*, 1982; Dunn and Raftery, 1982a,b; Raftery *et al.*, 1983; Dunn *et al.*, 1983; Dunn and Raftery, 1993).

5-(iodoacetamido)salicylic acid (IAS) and IANBD are two sulfhydryl-selective fluorescence probes that can be covalently bound to the *Torpedo* nAchR without apparent influence on the ligand binding and functional properties of the receptor measured *in vitro* (Dunn *et al.*,1980; Blanchard *et al.*,1982; Dunn and Raftery, 1982a,b, 1993). IAS can be reacted with the reduced disulfide α-Cys-192/193 which is close to the high-affinity binding sites. Binding of agonists and competitive antagonists could produce a saturable enhancement of IAS fluorescence, and dissociation constants obtained from equilibrium fluorescence titrations were similar to those obtained in direct radiolabeled binding studies with desensitized nAchR. Therefore, these changes in the fluorescence of bound IAS reflect conformational transition of the receptor-ligand complex at equilibrium and not the formation of the initial complex. Local anaesthetics, which are noncompetitive inhibitors (NCIs) of nAchR, did not cause any change in IAS fluorescence (with one exception, lidocaine, Dunn *et al.*, 1981). Although IANBD also reacts with sulfhydryl groups, there is no evidence that it reacts with the same cysteine residues as IAS (Dunn and Raftery, 1993).

Reaction of AchR with IANBD resulted in significant incorporation of the label into the α, β and γ subunits (Conti-Tronconi *et al.*, 1982). Under equilibrium conditions, there were no changes in NBD (nitrobenz-2-oxa-1,3-diazole) fluorescence as a consequence of the saturation of high-affinity sites by agonist binding. However, it was specifically and saturably enhanced upon the binding of relatively high concentrations of agonists (10^{-5} to 10^{-2} M) due to binding to distinct low affinity sites. Moreover, the dissociation constants obtained in agonist-induced NBD fluorescence titration were similar to those obtained in flux experiments for a wide range of agonists, e.g. 100 μM for Ach and 1 mM for Carb (Dunn and Raftery, 1982b; Raftery *et al.*, 1984, 1985). Also, these agonist-induced IANBD fluorescence changes were unaffected by physiologically relevant concentrations of d-TC or NCIs but were completely inhibited by preincubation of nAchR with saturating concentrations of α-BuTx. Thus, the binding sites with low affinity detected by IANBD appear to be distinct from the high-affinity sites monitored by IAS, and the binding to these low-affinity sites could be inhibited by α-BuTx but unaffected by d-TC ot NCIs. The stoichiometry of low-affinity sites was suggested to be two per receptor molecule, indicating that there exists at least four agonist binding sites per nAchR. The observation that the nAchR molecule contains four binding sites for α-dendrotoxin (α-Dtx) further supported this multisite theory (Conti-Tronconi and Raftery, 1986). The two low-affinity sites showed similar affinity for some agonists, e.g. Ach and Carb; however, SdCh was observed to exhibit two distinct affinities and only the higher affinity site was suggested to be involved in receptor activation (Dunn and Raftery, 1993).

Stopped-flow studies of agonist binding to receptor preparations that had been doubly

labeled by both IAS and IANBD fluorophores demonstrated that the conformational changes detected by IAS (equilibrium K_d~ 15 nM for Ach) occurred on slow time scales of seconds to minutes. This was too slow to account for channel activation, but may reflect receptor desensitization because of this long time duration. In contrast, a much faster conformational change was observed by change in IANBD fluorescence (K_{app} ~600 sec^{-1} and K_d~ 100 μM for Ach), which was more consistent with its possible involvement in rapid channel activation.

Therefore, a model (Raftery *et al.*, 1983, 1985) has been proposed in which the interaction of agonists with transient high concentration in the synaptic cleft to the low-affinity binding sites may induce rapid conformational changes of the receptor and channel opening. After the two high-affinity sites, located at the interfaces of α and non-α subunits, have been occupied by agonist in a longer time scale; considerably slower conformational changes are evoked. This is proposed to cause channel closing and receptor desensitization. According to this model, receptor activation and desensitization are parallel pathways rather than sequential ones.

It was previously reported that eserine, a reversible cholinesterase inhibitor, had depolarizing effects through direct interaction with the nAchR on cultured skeletal muscle cells (Harvey and Dryden, 1974a). From studies of ligand-induced ion flux into AchR-rich membrane vesicles and of equilibrium binding, Okonjo *et al.* (1991) reported that eserine could modulate ion flux in *Torpedo* nAchR, and this flux response was not affected by d-TC, α-BuTx, mAbs, or Ach-induced receptor desensitization. These data implied that eserine may bind to an adjacent but separate site from those for natural and synthetic cholinergic

ligands. Furthermore, eserine, like conventional agonists, was observed to induce a saturable enhancement of the fluorescence of NBD-labeled receptor which, as described above, appears to be a characteristic of channel activation. However, unlike other agonists, this eserine-induced NBD-fluorescence enhancement was not abolished by α-BuTx (Dunn and Raftery, 1993). Thus, it seems that eserine interacts with the nAchR at distinct site from both high- and low-affinity sites of the cholinergic ligands, but induces ion translocation by a common pathway.

Taken together, these results have implications for the ligand-binding on the nAChR. The two high-affinity binding sites for agonists and antagonists at the equilibrium are assumed to form an inter-subunit gorge or pocket between α/γ and α/δ subunit interfaces. Each of them may contain two subsites which are mutually exclusive at equilibrium. Agonist-induced IAS-fluorescence enhancement reflects occupancy of the two high-affinity sites and conformational changes of the receptor to the desensitized state. In addition, two low-affinity binding sites were also identified and postulated to be associated with channel activation. Occupancy of low-affinity sites by agonists, but not antagonists, results in an NBD-fluorescence enhancement that reflects rapid conformational changes. Therefore, it appears that each AchR molecule must carry at least four binding sites for cholinergic agonists. All four sites have been shown to inhibited by α-BuTx whereas d-TC antagonists bind to only the two high-affinity sites in the desensitized state. As described above, a fifth site, which is eserine sensitive but α-BuTx insensitive, has also been identified.

1. 4. 6. Binding Sites for Noncompetitive Inhibitors (NCI):

In both native membranes and reconstituted receptors, the ionic response is inhibited

by a class of particularly potent pharmacological agents, NCIs (Huganir *et al.*, 1979; Wu and Raftery, 1981; Popot *et al.*, 1981). It has been postulated that these agents may interfere directly or indirectly (or both) with the ion channel, although the detailed mechanisms of their actions are not fully understood. NCIs bind to different sites from Ach and provide a means to investigate structural aspects of the ion channel. Two categories of binding sites on nAchR for NCIs have been found: a single high-affinity, HTX (histrionicotoxin)-sensitive site, which is postulated to be located in the ion channel; and a population (10-20) of low-affinity, HTX-insensitive sites, which are suggested to be located at the lipid-protein interface (Changeux *et al.*, 1984; Ochoa *et al.*, 1989).

The effect of NCIs, including HTX, phencyclidine (PCP) and local anaesthetics, on receptor desensitization has been well studied. All these compounds were found to stabilize the desensitized state of the nAchR. Occupancy of the high-affinity site by NCIs is agonist-dependent and would consequently occlude the channel by simple steric hindrance. A less-appreciated effect of NCIs binding to this site is that they also increase the nAchR affinity for agonists as resulting from the stabilization of the desensitized state. NCIs binding to those low-affinity sites has also been shown to enhance the nAchR affinity for agonists and accelerate desensitization. Thus, the actions of NCIs can be considered in two different mechanisms: blocking the ion channel directly and accelerating the desensitization process (Ochoa *et al.*, 1989).

1. 5. Function of Other Subunits

In a series of experiments, different properties of conductance and gating due to differences in the structure of adult and embryonic mammalian receptors were demonstrated

(Takai *et al.*,1985; Mishina *et al.*, 1986). In bovine muscle, two types of nAchR have been identified, an adult junctional form with a large conductance (60 pS) and short channel open duration, and a fetal or denervated adult muscle receptor with lower conductance (40 pS) and a two- to three-fold longer current duration compared to that of adult AchR. In the fetal AchR, the subunit stoichiometry is $\alpha_2\beta\gamma\delta$. However, the γ subunit is substituted by a novel ϵ subunit in the adult $\alpha_2\beta\epsilon\delta$ AchR. This subunit switching was therefore suggested to be important in determining the different conductance and gating characteristics for different receptor types (Mishina *et al.*,1986). A more recent study reported that major determinants of this difference in conductance are located in the M2 segment of both the γ and ϵ subunits. The key differences are an exchange of amino acids in M2 and the M2 flanking region of both subunits (Herlitze *et al.*, 1996).

In addition, two forms of the γ-subunit have been found in the myogenic C2C12 cell line (Mileo *et al.*, 1995), one identical to the previously cloned γ-subunit (Yu *et al.*,1986) and the other lacking 52 amino acids within the N-terminal. This short γ (γ_s) subunit appears to be assembled into a functional AchR in heterologous systems (Fucile *et al.*,1996). It was observed that the γ_sAchR had a 4-fold longer open time than the γAchR. The regulation of γ and γ_s expression may, therefore, contribute to the modulation of channel open time during *in vivo* myogenesis (Grassi *et al.*,1998).

Subunit function has also been studied in chimeric experiments. These studies showed that δ subunit may be involved in the channel closure and subsequently determine the channel open time whereas the α subunit may mainly determine channel opening or agonist dissociation (Sakmann *et al.*, 1985). In a single-channel study of homologous and

hybrid receptors combined from *Torpedo* and mouse, Yu *et al.* (1991) proposed that γ and δ subunits may play a role in determining the single-channel conductance while the α and β subunits may not; it was also evident that α and δ subunits independently lengthen the channel open time with roughly equal effects. The β subunit, on the contrary, seemed to shorten the open time and the γ subunit had less influence on the channel open time.

No clear picture of the role of individual subunits in the gating properties of the nAchR has emerged from the above studies, but several subunits, not just one, may be influential in regulating channel activity.

1. 6. Ion Channel

1. 6. 1. Location of the Ion Channel

It has been widely accepted that the putative transmembrane domain, M_2 of each subunit, contributes to the lining of the ion channel. In addition to nAchR, M_2 segments of $GABA_A$ and glycine receptors have also been implicated in ion channel formation (Miller, 1989). Most residues of M_2 lining the channel are hydrophobic with negatively charged residues located at either end of the domain. These negatively charged residues were suggested to determine the ion conductance and selectivity (Imoto *et al.*, 1986, 1988; Changeux *et al.*, 1992; Changeux, 1995) and form three rings, one at the C-terminal end of the M_2 helix, one on its cytoplasmic side and one midway between the two, named the outer, the inner, and the intermediate ring, respectively. Furthermore, three more rings incorporating threonine, serine and leucine residues from the N-terminal to the C-terminal of M_2 were identified as potentially lining the channel (Giraudat *et al.*,1986; Hucho *et al.*,1986; Revah *et al.*, 1990; White and Cohen, 1992). In a site-directed mutagenesis study,

Leonard *et al.* (1988) reported that replacing conserved serine residues in this domain would significantly change the equilibrium binding affinity of QX-222, a quaternary ammonium open channel blocker.

1. 6. 2. Gating of the Ion Channel

Activation and channel opening of nAchR is an all-or-none event taking place within 0.3 msec after agonist release. This gating velocity comes from at least two general hypotheses: 1) all subunits surrounding the channel may undergo some global conformational rearrangements that subsequently open the channel, or 2) gating is electrostatic in that opposite charges would induce tight ion binding within the pore (Stroud *et al.*, 1990). Recent studies contradict the latter idea but cannot completely rule it out. Unwin (1993,1995) postulated that the gate was formed by a kink in the middle of the each M_2 domain formed by the Leu-251 residues. In the closed pore, these leucine residues may project inwards and associate in a tight ring to prevent the ions from passing through. By comparing the channel radius in the open and closed states of the homopentameric $\alpha 7$ receptor, Sankararamakrishnan *et al.*(1996) suggested that the minimum radius of the closed pore is approximately 2 Å and occurs in the vicinity of the leucine residue. This does not completely occlude the channel but effectively prevents sodium ions from permeating. Agonist binding to their binding sites would allosterically trigger small rotations of the M_2 helices allowing these gate-forming leucine side chains to be driven away from the central axis to create an open pore with a minimum radius of approximately 6.5 Å (Hille, 1992). This open pore size is large enough for monovalent, Na^+ ions in particular, and divalent cations as well as for some small positively-charged organic molecules to pass through the

channel. It has been reported that there is a distance about 20 Å between the agonist binding site and the channel (Machold *et al.*, 1995; Hucho *et al.*, 1996). To be such a short distance, binding of agonist could rapidly result in transient channel opening. During desensitization, these residues would swing back into the lumen of the channel to form the nonconducting state. Recent mutagenesis studies reinforced the idea that each of the leucine residues participate independently and symmetrically in a key step in the structural transition between the closed and open states (Labarca *et al.*, 1995) and agonist-receptor binding (Filatov and White, 1995; Kearney *et al.*, 1996). However, by using a substituted cysteine accessibility technique, Akabas *et al.* (1994) suggested that the channel gate is at least as close to the cytoplasmic side as Glu-241.

1. 7. Desensitization of the Receptor

Desensitization of nAchR at the neuromuscular junction was first described by Katz and Thesleff (1957). It is an intrinsic molecular property of the nAchR which is only triggered by agonists. Upon prolonged exposure to an agonist, the receptor undergoes conformational rearrangements of its structure which leads to channel closing, loss of the permeability response and meanwhile an increase in affinity of the receptor for its agonist.

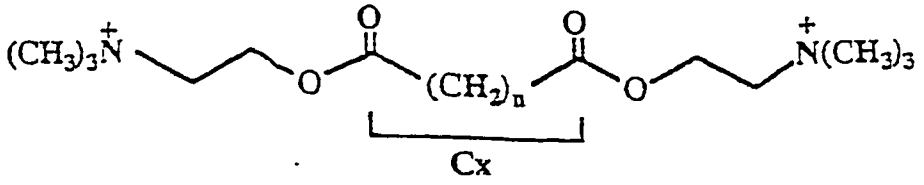
These are at least two kinetic forms of desensitization: a fast-onset form occurring in the millisecond time scale followed by a slow inactivation one exhibiting in the second to minute time scale, both of which are associated with the conformational changes and the elevation of the affinity for agonists (Ochoa *et al.*, 1989; Stroud *et al.*, 1990; Dunn, 1993; Wu and Miller, 1994). Recovery of the desensitized receptor to its resting lower affinity state occurs only slowly, over time scales of minutes, upon removal of agonist.

Although the physiological significance of nAchR desensitization is not yet thoroughly understood, existing evidence suggests that it may represent a general protective mechanism that prohibits the receptor from an over-exposure to stimuli by modulating the frequency of synaptic response. Thus it may play a role in cellular homeostatic process (Ochoa *et al.*, 1989). A noteworthy property of nAchR desensitization is its capacity to be modulated: high concentrations of agonist, membrane potentials, divalent cations, NCIs, neuropeptides and receptor phosphorylation have all been implicated in the regulation of receptor desensitization (Ochoa *et al.*, 1989). This controlled rate of desensitization was considered to reflect a functional alteration of the amount of binding energy effectively relayed to channel opening, both in terms of open time and channel conductance (Stroud *et al.*, 1990).

2. Specific Objective

Two subsites within each high affinity binding site of the nicotinic receptor have been recently suggested by the results of dissociation and association studies (Dunn and Raftery, 1997 a, b). One is the primary site and the other the secondary. At low concentrations, one molecule of monoquaternary agonist binds to the primary site. At higher agonist concentrations, the secondary site can be occupied and this occupancy significantly accelerates the dissociation of the first molecule from the primary site. At the equilibrium of the experimental conditions, only one site at a time is occupied. However, a large bischoline agonist can bridge these two subsites provided its length is long enough. This would prevent a second bischoline molecule from binding.

In this present study, the two-subsite model has been further examined. In order to determine the existence and distance between the two subsites, a series of bis-quaternary cholinergic agonists (Figure 4) with the number of methylene groups between the two carbonyl groups increasing from 0 to 10 was used. Each of the two quaternary amines is assumed to be able to bind to distinct subsites. When the number of methylene groups increases, the chain length of these bischolines simultaneously increases. We have measured the affinities of these bis-quaternary ligands for the nAchR by their abilities to displace either radiolabeled Ach or SdCh, and by directly measuring their affinities using fluorescence techniques. It is hypothesized that the affinities of the bischoline ligand will increase when it is able to span the two subsites. By comparing the affinities of this bis-quaternary series, a possible distance between the two subsites has been estimated.



Bischoline esters	Cx	n
Oxalyldicholine (OxdCh)	C2	0
Malonyldicholine (MadCh)	C3	1
Succinyldicholine (ScdCh)	C4	2
Glutaryldicholine (GldCh)	C5	3
Adipyldicholine (AddCh)	C6	4
Pimelyldicholine (PidCh)	C7	5
Suberyldicholine (SdCh)	C8	6
Azelyldicholine (AzdCh)	C9	7
Decyldicholine (DedCh)	C10	8
Dodecyldicholine (DodCh)	C12	10

Figure 4. The nomenclature and structure of the series of bis-quaternary ligands.

3. Materials and Methods

3. 1. Materials

The electric organs of *Torpedo California* were obtained from Pacific Biomarine (San Pedro, CA). Carbamylcholine chloride, acetylcholine chloride, succinyldicholine dichloride (C4; SudCh), suberyldicholine dichloride (C8; SdCh), diethyl-*p*-nitrophenylphosphate (DNPP), DTT, α-bungarotoxin, L-α-phosphatidyl-choline (L-α-lecithin), 3-[(3-cholamidopropyl)-dimethylammonio-]1-propane-sulfonate (CHAPS) and bovine serum albumin (BSA) were purchased from Sigma Chemical Co.. [^{3}H]Acetylcholine chloride (specific activity = 55.2 mCi/mmol) was purchased from DuPont-NEN Canada. [^{3}H]Suberyldicholine diiodide (specific activity = 50.85 mCi/mmol) was synthesized as described previously (Dunn and Raftery 1997a). Oxalyldicholine (C2; OxdCh), malonyldicholine (C3; MadCh), glutaryldicholine (C5; GldCh), adipyldicholine (C6; AddCh), pimelyldicholine (C7; PidCh), azelyldicholine (C9; AzdCh), decyldicholine (C10; DedCh), dodecyldicholine (C12; DodCh), 5-IAS and arecolone methyliodide (arecolone Met) were kindly provided by Dr. Michael A. Raftery (University of Minnesota, St Paul, MN)

3. 2. Methods

3. 2. 1. Preparation of nAchR-Enriched Membrane Fragments:

Membrane fragments enriched in nAchR were prepared from frozen *Torpedo* electric organ as previously described (Elliott *et al.*, 1980). Most procedures of membrane isolation consist of repeated cycles of homogenization and centrifugation to obtain a fraction of higher nAchR content. To prevent protein degradation, all procedures were carried out at 4°C and

protease inhibitors EDTA (5 mM) and phenylmethylsulfonyl fluoride (PMSF 1 mM) were routinely included in the buffers. Thus, activities of Ca^{2+}-dependent protease and serine protease were reduced. Since PMSF is relatively unstable in solution, it was added to the homogenization buffer immediately prior to use. Sodium azide (0.02% w/v) was also added as an anti-bacterial reagent.

Torpedo electric organs were removed from -86°C and shattered into small fragments within a plastic bag using a hammer. The fragments were suspended and homogenized in an equal volume of homogenization buffer (10 mM sodium phosphate, pH 7.4, 400 mM NaCl, 5 mM EDTA, 0.02% NaN_3). The homogenization sequence involved two periods of high speed grinding, each lasting 1 min with 1 min rest between, using a commercial Waring blender. The aliquots of homogenate were then reground for four periods of 30 sec (with a 30 sec interval between each period) at setting of 3 in a Virtis 45 homogenizer. Connective tissue, nuclei and other large particles were pelleted by low speed centrifugation of the homogenate for 10 min at 2,000g in a Sorvall GSA rotor. The supernatant was filtered through four layers of cheesecloth and recentrifuged for 1 hr at 45,600g in a Beckman Ti45 rotor. Following the centrifugation, the supernatant was discarded and the pellet was resuspended in about 40 ml low salt buffer (10 mM sodium phosphate, pH 7.4, 1 mM EDTA, 0.02% NaN_3) to provide osmotic shock and further subjected to homogenization for two periods of 30 sec at setting of 3 in the Virtis 45 homogenizer, with 30 sec interval in between each. Sucrose and NaCl were then added to final concentrations of 30% (w/w) and 400 mM (2.3% w/w) respectively and the mixture was gently stirred for 20 min at 4°C to allow the sucrose to dissolve.

To prepare a sucrose gradient in a Beckman Vti50 tube, 5 ml 50%, 5 ml 39% and 12 ml 35% sucrose (all w/w) in buffer B (10 mM sodium phosphate, pH 7.4, 400 mM NaCl, 1 mM EDTA, 0.02% NaN_3) were layered from the bottom of the tubes respectively to make a discontinuous sucrose gradient. Samples of 15 ml homogenate were applied above the 35% sucrose layer and further overlaid with buffer B to fill each tube to the top. The tubes were centrifuged at 163,000g for 1 hr with a Beckman Vti50 rotor with slow acceleration and deceleration in the range of 2,000 rpm. After centrifugation, three bands were observed (heavy bands at the top and middle of the gradient and a lighter one near the bottom). The upper band was discarded and the middle layer collected and diluted by a factor of about 2-3 with ice cold buffer B. The final centrifugation was performed for 1 hr at 45,600g in a Beckman Ti45 rotor. The pellet was resuspended in about 15 ml of resuspension buffer (10 mM Tris-HCl, pH 7.4, 0.2 M NaCl, 0.02% NaN_3) in the Virtis 45 homogenizer. The membranes were quickly frozen in liquid nitrogen and stored at -86°C for future use.

3. 2. 2. Preparation of Alkali-Extracted nAchR Membranes:

This partial purification of nAchR receptor was performed as previously described (Neubig *et al.*,1979; Moore *et al.*,1979). The crude *Torpedo* membranes were thawed and diluted 20-fold into distilled water at room temperature. 20 µl aliquots of 0.2 N NaOH were added with rapid mixing until the pH was adjusted to 11. The mixture was gently stirred at 4°C for at least 15 min and then centrifuged for 45 min at 15,600g in a Sorvall SS34 rotor. The supernatant was carefully discarded and the pellet gently rinsed with a small amount of buffer and then resuspended in 1 ml of Ca^{2+}-free *Torpedo* physiological saline (Ca^{2+}-free *Torpedo* Ringers, 20 mM Hepes, pH 7.4, 250 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 0.02%

NaN_3). The membranes were stored at 4°C for future use.

3. 2. 3. Equilibrium Binding Assay

3. 2. 3. 1. Equilibrium Binding of [^{3}H]Ach and [^{3}H]SdCh to Membrane-Bound nAchR Receptor:

Measurements of the binding of [^{3}H]Ach and [^{3}H]SdCh to *Torpedo* crude membrane preparations were performed by centrifugation assays. AchE activity was first inhibited by the addition of 1/200 volume of a 300 mM stock of diethyl-p-nitrophenylphosphate (DNPP, Sigma) in 2-propanol to a concentrated membrane suspension (approximately 5 mg protein/ml). After 3 min incubation at room temperature, the DNPP-treated membranes were diluted about 10-fold in *Torpedo* physiological saline (20 mM Hepes, pH 7.4, 250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 0.02%NaN_3) and stored on ice until use. Aliquots of membranes (250 µl, 0.2 mg protein/ml) were added to various concentrations of [^{3}H]Ach or [^{3}H]SdCh ranging from 0 to 1 µM to give a final concentration of membranes of 0.1 mg protein/ml. Following 45 min incubation at room temperature, 50 µl aliquots were taken to estimate the total radiolabeled ligand concentration directly. After centrifugation in a microcentrifuge (Eppendorf 3200) at 13,000 rpm for 15 min at 4°C, 50 µl aliquots from the supernatant were removed for determination of free radiolabeled ligand. 5 ml scintillation fluid (Cytoscint ICN) were added to each sample which were then counted for [^{3}H] using a Beckman LS 6500 Scintillation Counter. The amount of bound radiolabeled ligand was calculated by subtracting free from total ligand concentration. Non-specific binding was determined from parallel measurements of binding in the presence of excess unlabeled ligand of 1 mM Ach or 1 mM SdCh.

3. 2. 3. 2. Competitive Binding Assays:

Equilibrium displacement experiments were carried out by the centrifugation assay. Aliquots of DNPP-treated *Torpedo* membranes (150 µl 0.2 mg protein/ml) were incubated with either [^{3}H]Ach or [^{3}H]SdCh and increasing concentrations of C_2 to C_{12} (0-1 mM) to give a final concentration of protein of 0.1 mg/ml and radiolabeled ligand of 0.2 µM in a total volume of 300 µl in *Torpedo* physiological saline. After 30 min incubation at room temperature, 50 µl aliquots were removed to determine total ligand concentration. The samples were microcentrifuged (Eppendorf 3200) at 4°C at 13,000 rpm for 15 min. 50 µl aliquots from the supernatant were taken for estimation of free ligand concentration.

3. 2. 4. Fluorescence Titration Experiments

3. 2. 4. 1. Fluorescence Labeling of nAchR-Enriched Membrane Fragments:

Covalent modification of the membrane-bound receptor by 5-(iodoacetamido)salicylic acid (IAS) was carried out as previously described (Dunn *et al.*, 1980). Briefly, the pH-treated membrane fragments were diluted to provide a concentration of 0.3-0.5 mg/ml in Ca^{2+}-free *Torpedo* physiological saline which had been previously flushed with nitrogen for 15 min. Dithiothreitol (DTT) was added to a final concentration of 50 µM from 30 mM stock and the reduction was allowed to proceed for 1 hr at 4°C. IAS was added from a stock concentration of 25 mM in 200 mM Tris-HCl, pH 8.5, to a final concentration of 250 µM. The reaction mixture was readjusted to pH 7.4, shielded from light and continuously stirred for 2 hr at 4°C. The mixture was then diluted with the same buffer and centrifuged at 15,600g for 45 min in a Sorvall SS45 rotor. The pellet was resuspended in about 50 ml of the same buffer using the Virtis 45 homogenizer at setting 3 for four bursts

of 20 sec with 20 sec interval between. The homogenate was centrifuged as above. The pellet was finally resuspended in a small volume of the same buffer to give a final concentration of 3~5 mg protein/ml.

3. 2. 4. 2. Equilibrium Fluorescence Measurements:

The binding of agonist to IAS-labeled membrane preparations was monitored by changes in IAS fluorescence, as measured by energy transfer from the receptor protein using an excitation wavelength of 280 nm with an excitation slit width of 4 nm and an emission wavelength of 430 nm with an emission slit width of 10 nm (Dunn *et al.*,1980). Fluorescence spectra were recorded on a Perkin-Elmer MPF-4 fluorimeter fitted with a magnetically stirred and thermostatically controlled cuvette holder. All measurements were carried out at 25°C, and after the ligand addition, the samples were continuously stirred for at least 2 min before their fluorescence was measured.

A fluorescence titration of carbachol (Carb) binding was first carried out as the standard. Membrane samples were diluted in *Torpedo* physiological saline at room temperature to a final volume of 2 ml in a quartz cuvette. 5 µl Aliquots of Carb solutions in *Torpedo* physiological saline were repeatedly added to the sample and the fluorescence measurements were recorded after 2 min stirring. In order to minimize photolytic reactions, all samples were shielded from light between measurements. Small corrections for non-specific effects were carried out by using the results of a parallel titration of IAS-labeled AchR which had been preequilibrated with 2 µM α-BuTx. The final concentration of Carb was 10 µM and of membrane was 0.1 mg protein/ml. The fluorescence titration measurements of each bischoline were performed in steps similar to Carb and the final

concentration of each bischoline was 9.5 μM. Scanning of the changes in IAS fluorescence induced by Carb and the bischoline ligands was carried out over the emission spectrum of 400 to 500 nm before and after addition of drugs.

3. 2. 5. Protein Assay

Protein concentrations were estimated using the Bio-Rad Protein Assay (Bio-Rad Laboratories) with bovine serum albumin (BSA) as a protein standard. Diluted samples or 0.1 mg/ml BSA in distilled water were prepared in a final volume of 800 μl containing approximately 0-15 μg protein. Bio-Rad reagent (200 μl) was added to each sample. After mixing and incubation at room temperature for 5 min, the absorbance at wavelength of 595 nm (A_{595}) was measured using a Beckman DU-40 spectrophotometer.

3. 2. 6. Data Analysis

All data of equilibrium binding assays and equilibrium displacement assays were analyzed using Inplot 4 (Version 2.1; GraphPad Software, San Diego, CA). Equilibrium binding data of both [^{3}H]Ach and [^{3}H]SdCh were analyzed either by Scatchard plots or by a simple binding isotherm:

$$[RL]=[R_o][L]/(K_d+[L])$$

where [RL] represents the occupied receptor, [R_o] is the total concentration of receptor binding sites and [L] if the free ligand concentration. The Scatchard plot is simply a linear function demonstrating the relationship between [bound]/[free] versus [bound] based on the equation:

$$[RL]/[L] = [R_o]/K_d - [RL]/K_d$$

The slope of this straight line is $-1/K_d$ and the intercept on the x-axis is R_o. The

competition plots are fitted by sigmoid curves using the equation:

$$Y = A + (B-A)/(1+10^{X-C})$$

where Y is the measured binding activity of [^{3}H]-ligand, X is the log of the concentration of bischoline ligands, A is binding activity when maximum displacement by bischoline ligands occurs, B is the measured radioligand binding in the absence of competing ligand and C is logIC_{50}.

All data from fluorescence titration experiments were analyzed by GraphPad Prism (Version 2.0; GraphPad Software Inc, San Diego, CA). Since IAS monitors high-affinity binding and there is significant depletion of added ligand due to binding, fluorescence titrations were fit by non-linear regression models by the equation (Dunn and Raftery 1997a):

$$Fl = F_{RL}[RL] = 0.5\ F_{RL}\ \{(L_o + R_o + K_d) - [(L_o + R_o + K_d)^2 - 4\ R_o\ L_o]^{1/2}\}$$

where Fl is the observed fluorescence (after subtraction of the fluorescence of IAS-labeled Ach receptor alone), L_o is the concentration of added bischoline ligands, R_o is the concentration of binding sites, K_d and F_{RL} are the dissociation constant which need to be determined and fluorescence enhancement per unit concentration of RL complex, respectively. At saturation, the maximum observed fluorescence enhancement (F_{max}) is given by:

$$F_{max} = F_{RL}[R_0]$$

4. Results

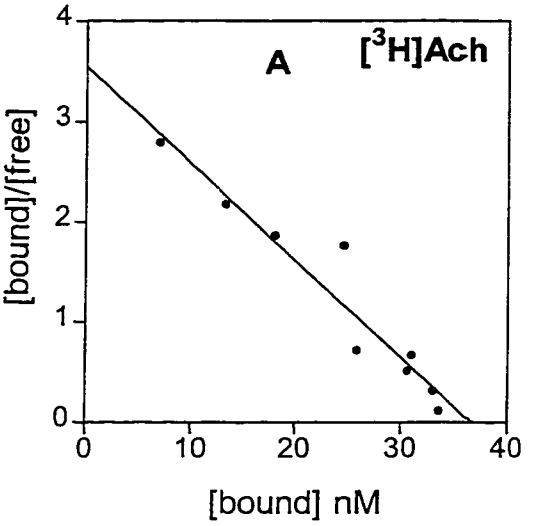
4. 1. Equilibrium Binding of Radiolabeled Ach and SdCh to Crude *Torpedo* Membranes:

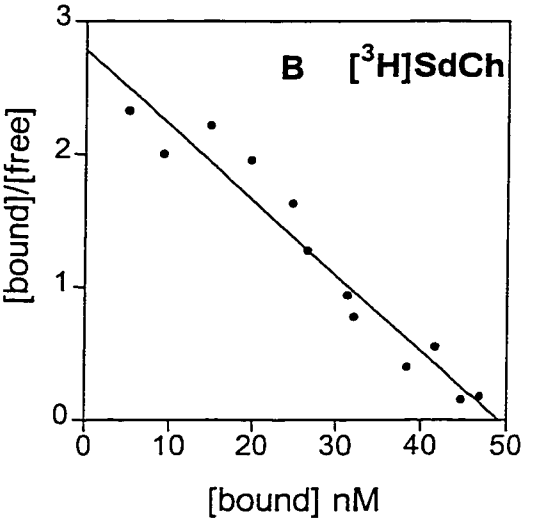
The high-affinity binding of [^{3}H]Ach and [^{3}H]SdCh to crude membrane-bound nAchR has been determined by using centrifugation assays. In these assays, a constant amount of membranes was incubated with increasing concentrations of radioligand until equilibrium was reached and free and bound radiolabeled ligands were then physically separated in order to estimate the amount of bound radioligand. In the present study, the equilibrium binding of [^{3}H]SdCh was found to be similar to the binding of [^{3}H]Ach which is in good agreement with previous studies (Dunn and Raftery, 1997 a,b). Scatchard plot analysis (Figure 5) indicated that binding of [^{3}H]SdCh was to an apparently homogenous population of sites with a equilibrium dissociation constant (K_d) of 14.9±2.5 nM (mean ± S.D.) and maximum receptor binding sites (B_{max}) of 0.4±0.1 nmol/mg protein (n=3). The binding of [^{3}H]Ach also showed a single class of binding site characterized by K_d and B_{max} of 11.4±1.9 nM and 0.3±0.04 nmol/mg protein, respectively (n = 4) which was consistent with many previous studies (Neubig and Cohen, 1979; Blanchard *et al.*,1982). As in many previous reports (Quast *et al.*,1979; Dunn *et al.*,1980; Blanchard *et al.*,1982; Dunn and Raftery, 1993, 1997a), we have found no evidence for cooperativity in the high affinity binding (Figure 5).

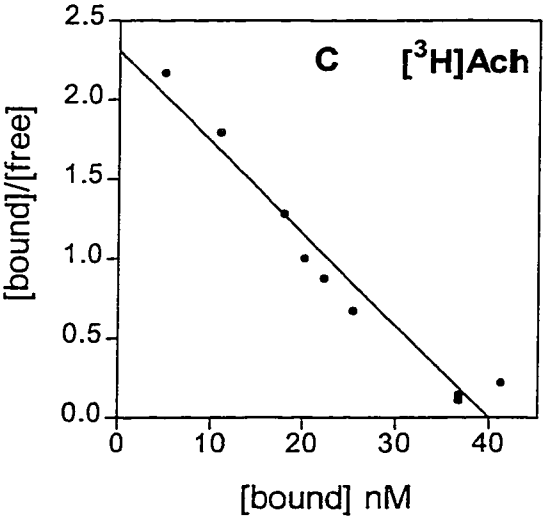
4. 2. Competitive Displacement of Radiolabeled Ach and SdCh by a Series of Bischolines:

In competitive displacement assays, the ability of bischoline ligands to displace

Figure 5. Scatchard plot analysis of binding of [^{3}H]Ach and [^{3}H]Sdch to *Torpedo* crude and IAS-labeled membrane fragments. Equilibrium Binding of [^{3}H]Ach and [^{3}H]SdCh were performed by the centrifugation assay as described in the method. Final concentration of nAchR was 0.1 mg protein/ml. The estimated maximum radioligand binding sites (B_{max}) and K_d for [^{3}H]Ach were 0.36 nmol/mg protein and 9.7 nM (A), and were 0.49 nmol/mg protein and 17.6 nM for [^{3}H]SdCh (B). After the alkali-treatment and IAS-labeling of the receptor membranes, the B_{max} and K_d values remained unchanged for [^{3}H]Ach binding of 0.4 nmol/mg protein and 17.2 nM (C). B_{max}s come from the receptor sites (R_o) divided by final membrane concentration (0.1 mg/ml).


A
[^{3}H]Ach
[bound]/[free]
[bound] nM
0
1
2
3
4
0
10
20
30
40


B
[^{3}H]SdCh
[bound]/[free]
[bound] nM
0
1
2
3
0
10
20
30
40
50


C
[^{3}H]Ach
[bound]/[free]
[bound] nM
0.0
0.5
1.0
1.5
2.0
2.5
0
10
20
30
40

radiolabeled Ach and SdCh from their specific receptor binding sites was investigated. Defining IC_{50} as the concentration of bischolines that displaced 50% of the radiolabeled agonist, an inhibitory equilibrium dissociation constant for the bischolines, K_i, was determined from the Cheng-Prusoff equation (Cheng and Prusoff, 1973):

$$K_i = IC_{50}/[1 + [L]/K_d]$$

where [L] is the concentration of radiolabeled ligand used in the displacement study and K_d is the dissociation constant of the radioligand obtained from the equilibrium binding assay (see above).

Calculated K_is for displacement of [^{3}H]Ach or [^{3}H]SdCh by Ach are shown in Table 1. The inhibitory dissociation constants were observed to be similar, i.e. $K_i = 15.7 \pm 2.1$ nM (n = 3) for [^{3}H]Ach displacement and $K_i = 17.1 \pm 1.0$ nM (n=3) for [^{3}H]SdCh displacement. Likewise, virtually identical values of 3.9 ± 1.4 nM and 4.4 ± 2.2 nM for unlabeled SdCh competition for [^{3}H]Ach and [^{3}H]SdCh sites respectively were obtained (Table 1).

As shown in Figure 6 and Table 2, various bischoline compounds were evaluated for their ability to compete for [^{3}H]Ach or [^{3}H]SdCh binding. All the bischoline ligands were shown to be able to displace the radioligands completely. Three groups of K_is, each of which shared similar values, were observed. OxdCh (C2), MadCh (C3) and SudCh (C4) (see Figure 4) had similar K_i values for displacement of [^{3}H]Ach, i.e.154.4 ± 34.3 nM (n = 4), 213.1 ± 23.2 nM (n = 4) and 167.3 ± 35.3 nM (n = 6), respectively. Addition of an additional one to three methylene groups leads to significant increase in affinity. Thus GldCh (C5), AddCh (C6) and PidCh (C7) showed similar K_i values for [^{3}H]Ach displacement, i.e. 33.7 ± 5.2 nM (n=3), 13.7 ± 3.0 nM (n=3) and 18.7 ± 7.8 nM (n=3), respectively. Addition of one or more

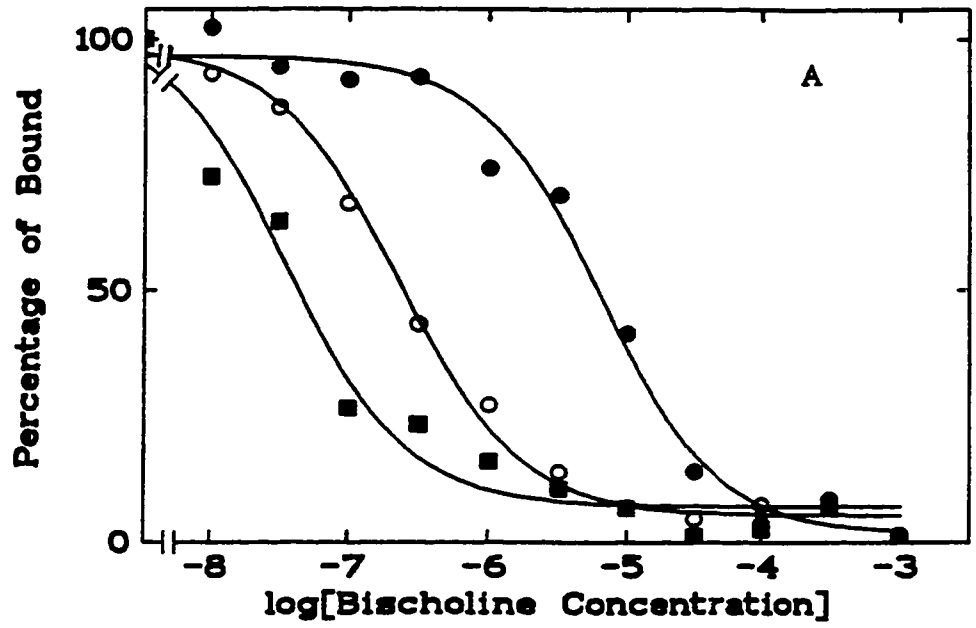
Radiolabeled ligand	Cold Ach displacement K_i [nM]	Cold SdCh displacement K_i [nM]
[^{3}H]Ach	15.7 ± 2.1	3.9 ± 1.4
[^{3}H]SdCh	17.1 ± 1.0	4.4 ± 2.2

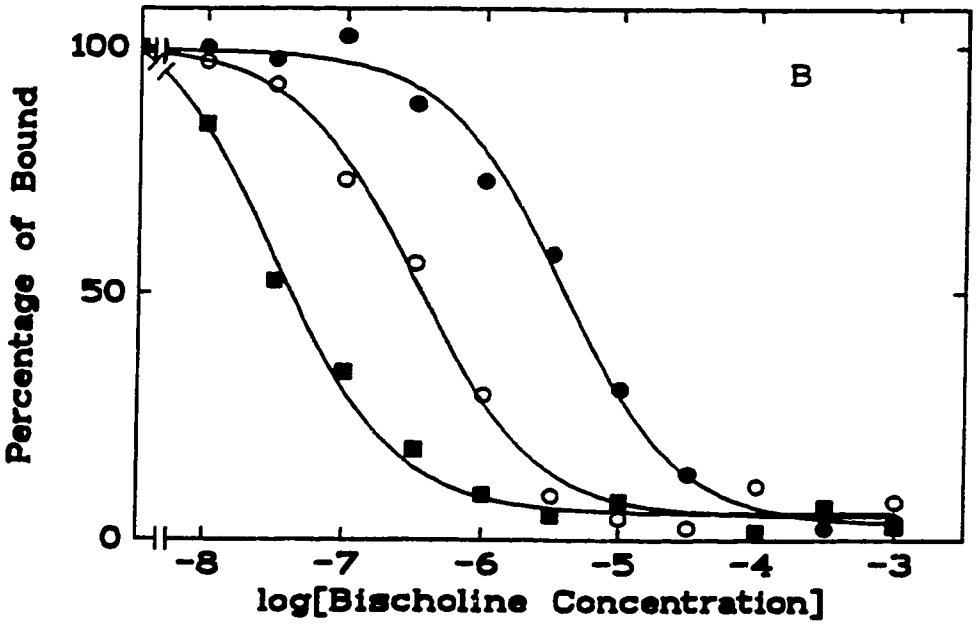
Table 1. Parameters of radiolabeled Ach and SdCh displaced by cold Ach and SdCh. Equilibrium displacement assay was carried out with *Torpedo* crude membranes using centrifugation method. The K_i values were calculated from the Cheng-Prusoff equation: $K_i = IC_{50}/(1+ [L]/K_d)$, where [L] was the concentration of radiolabeled ligand used in this displacement study (0.2 μM) and K_d for [^{3}H]Ach and [^{3}H]SdCh was11.39 nM and 14.92 nM, respectively. IC_{50} was defined as the concentration of bischoline esters that displaced 50% of radiolabeled ligands.

Bischoline esters (Cx)	[^{3}H]Ach displacement K_i [nM]	[^{3}H]SdCh displacement K_i [nM]	IAS titration K_d [μM]
OxdCh (C2)	154.4 ± 34.3	225.1 ± 48.4	1.3 ± 0.2
MadCh (C3)	213.1 ± 23.2	283.9 ± 78.1	1.3 ± 0.2
SudCh (C4)	167.3 ± 35.3	209.4 ± 52.6	1.2 ± 0.2
GldCh (C5)	33.7 ± 5.9	63.2 ± 8.2	0.3 ± 0.1
AddCh (C6)	13.7 ± 3.0	20.2 ± 3.4	0.2 ± 0.04
PidCh (C7)	18.7 ± 7.8	17.9 ± 2.8	0.3 ± 0.1
SdCh (C8)	3.9 ± 1.4	4.4 ± 2.2	0.3 ± 0.1
AzdCh (C9)	1.9 ± 0.7	2.2 ± 0.4	0.3 ± 0.1
DedCh (C10)	3.7 ± 1.4	3.6 ± 0.7	0.3 ± 0.02
DodCh (C12)	5.0 ± 1.9	2.5 ± 0.6	0.3 ± 0.1

Table 2. Affinity parameters for a series of bischoline esters from equilibrium displacement binding and fluorescence titration studies. Inhibitory equilibrium dissociation constant (K_i) of bischoline esters were obtained by competing the radiolabeled Ach and SdCh using the centrifugation assay. The IAS fluorescence was titrated with the bischoline esters using IAS-labeled *Torpedo* nAchR. The K_ds were calculated: $Fl = F_{RL}\,[RL] = 0.5\,F_{RL}\,\{(L_o + K_d + R_o) - [(L_o + K_d + R_o)^2 - 4\,R_o\,L_o]^{1/2}\}$, where Fl was the observed fluorescence, L_o was the added bischoline ligand concentration, R_o was the concentration of binding sites, and F_{RL} was the fluorescence enhancement due to the bischoline binding.

Figure 6. Representative equilibrium displacement experiments of radioligand [^{3}H]Ach and [^{3}H]SdCh, respectively by a series of bischoline esters performed by centrifugation assay. *Torpedo* crude membrane fragments were incubated with constant concentration of radiolabeled ligands of 0.2 μM and various concentrations of bischoline esters. The final membrane concentration was 0.1 mg protein/ml. (A) The equilibrium binding of [^{3}H]Ach was disturbed by the introduction of a range of concentrations of competitive ligands, MadCh (●), AddCh (○) and DedCh (■). The estimated results of K_is were 0.3 μM for MadCh (C3), 11.5 nM for AddCh (C6), 1.78 nM for DedCh (C10). (B) The equilibrium binding of [^{3}H]SdCh was displaced by SudCh (●), PidCh (○) and AzdCh (■), respectively. The values of K_is were: 0.234 μM for SudCh (C4), 20.34 nM for PidCh (C7), 2.11 nM for AzdCh (C9).


100
50
0
Percentage of Bound
A
-8
-7
-6
-5
-4
-3
log[Bischoline Concentration]


100
50
0
Percentage of Bound
B
-8
-7
-6
-5
-4
-3
log[Bischoline Concentration]

methylene groups from PidCh results in further affinity increase for SdCh and longer bischoline ligands with their inhibitory dissociation constants displaying similar magnitudes of 2-5 nM. Therefore, this equilibrium [^{3}H]Ach displacement experiment divided the affinity of the bischoline series into three groups: C2-C4 (K_i ~ 100 -200 nM), C5-C7 (K_i ~ 10-30 nM) and C8-C12 (2-5 nM); each group showed approximately 10-fold difference in affinity, i.e. C5-C7 were 10-fold more potent than C2-C4 but 10-fold less potent than C8-C12. Two clear breaks between C4/C5 and C7/C8 were observed. A virtually identical pattern was seen with the displacement of [^{3}H]SdCh by these bischoline compounds. The calculated K_is for [^{3}H]SdCh displacement were very close to those for [^{3}H]Ach displacement. Again, two clear breaks between C4/C5 and C7/C8 were observed (Table 2).

4. 3. Fluorescence Titration Experiments

4. 3. 1. Equilibrium Binding of Crude and IAS-labeled *Torpedo* Membrane with [^{3}H]Ach

The dissociation constant for Ach binding to IAS-labeled nAchR and number of binding sites were measured by a centrifugation assay with [^{3}H]Ach. The results shown in Figure 5 demonstrate that IAS-treatment did not alter the K_d nor the B_{max} for Ach binding, which was in good agreement with previous findings (Dunn *et al.*, 1980; Blanchard *et al.*, 1982; Dunn and Raftery, 1993, 1997b). No evidence for heterogeneity of binding sites was observed after IAS labeling.

4. 3. 2. Fluorescence Properties of IAS-treated Membrane Preparations

Using an excitation wavelength of 280 nm, the maximum fluorescence emission of IAS-labeled receptor membranes was observed to occur at 430 nm as shown in Figure 7.

Addition of 40 μM Carb led to a fluorescence enhancement of bound IAS and this increase in fluorescence was completely abolished by preincubation of the receptor with 2 μM α-BuTx. This is in good agreement with previous observations (Dunn *et al.*, 1980).

The results of a typical fluorescence titration of IAS-labeled membrane fragments by Carb are shown in Figure 8. A non-linear regression fit to this data provided a K_d value of 0.5 ± 0.1 μM.

4. 3. 3. Equilibrium Binding of Bischoline Compounds

The fluorescence of IAS-labeled nAchR was also enhanced upon addition of bischoline ligands and this enhancement was found to be saturable at high ligand concentration and to be completely blocked by prior incubation of IAS-labeled receptor with α-BuTx, suggesting that it was specific for the AchR. All the plots were fit by a non-linear regression techniques. Equilibrium dissociation constants obtained from fluorescence titrations by these bischoline compounds are listed in Table 2 and representative titrations are shown in Figure 8. Unlike the equilibrium displacement assays, the K_d values of these ligands from the fluorescence titration were clearly divided in two groups: C2-C4 (K_d~ 1.2 μM), C5-C12 (K_d~ 0.3 μM), with the C5-C12 group being approximately 4-fold more potent than C2-C4. A break between C4/C5 was obtained.

A comparison of inhibitory dissociation constants from equilibrium displacement assays and equilibrium dissociation constants from fluorescence titration assays is shown in Figure 9. The K_i and K_d values were plotted versus hydrocarbon chain length. As the alkyl group increases in size, both the K_i and K_d values decrease to different levels, indicating an increase in affinity to different extent for these bis-quaternary ligands. An affinity break

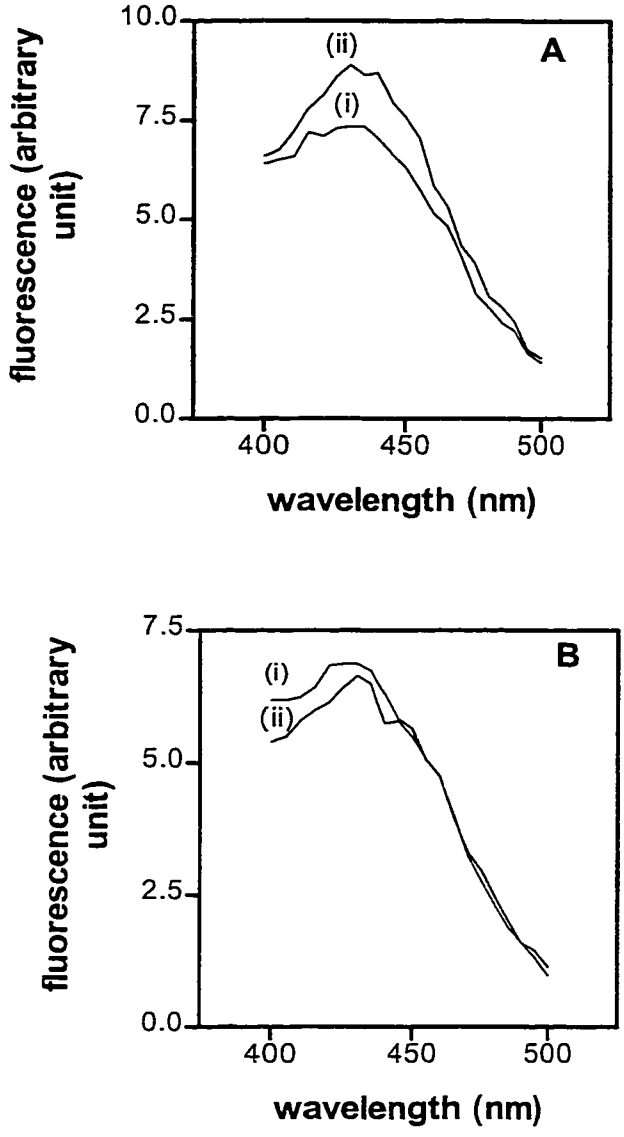
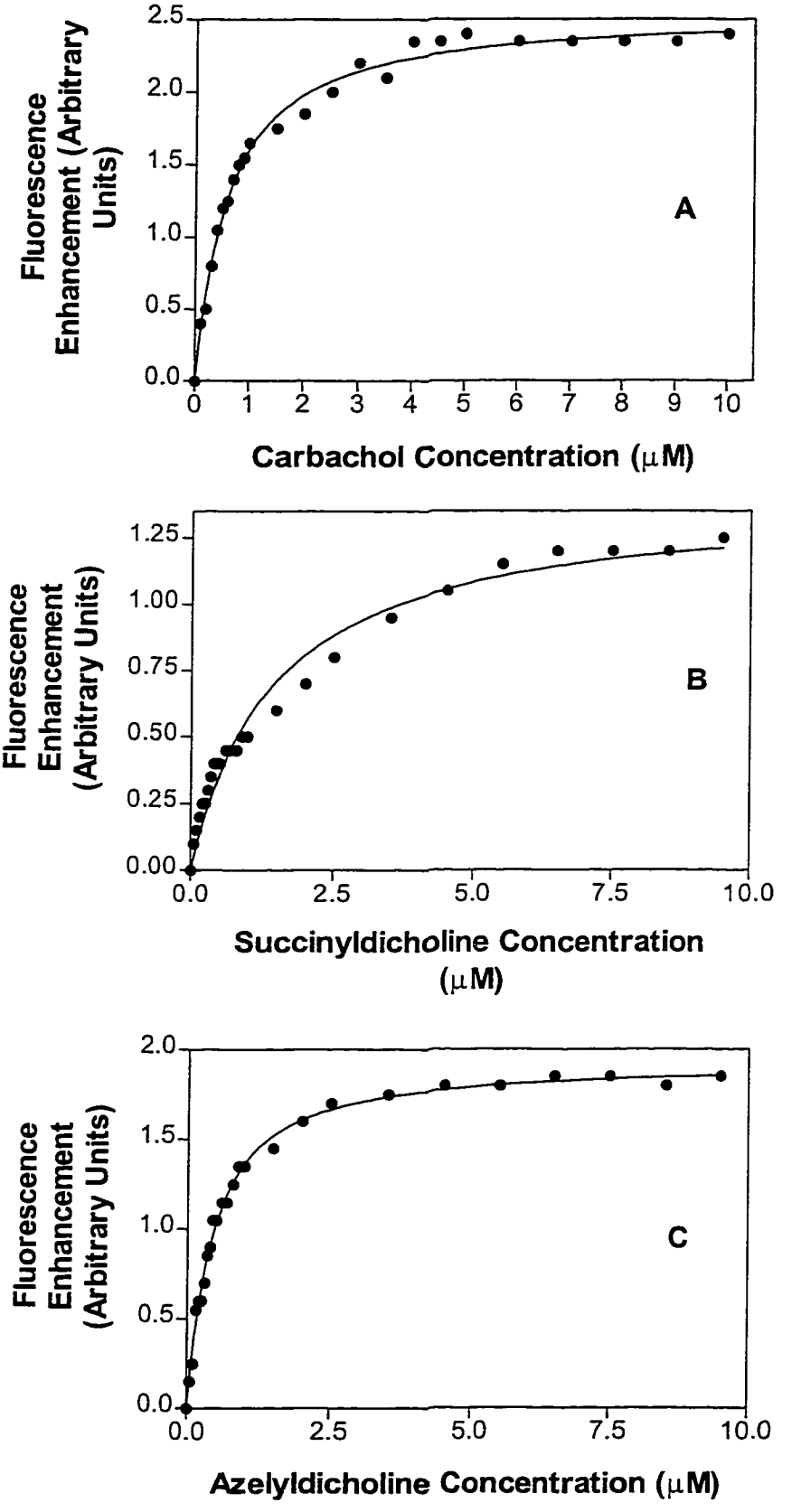


Figure 7. Fluorescence scanning spectra of IAS-labeled *Torpedo* membrane fragments. The nAchR concentration was approximately 0.1~0.2 mg protein/ml. By energy transfer fluorescence, the excitation spectra was at 280 nm and the emission peak occurred at 430 nm indicating that the emission fluorescence was detected at 430 nm. (A) The spectra were recorded in the absence (i) or in the presence of 40 μM carbachol (ii). (B) The IAS-labeled *Torpedo* membranes were preincubated with 2 μM α-BuTx before 40 μM Carb addition. The spectra were recorded in the absence (i) or in the presence of 40 μM Carb (ii).

Figure 8. Equilibrium fluorescence titration of IAS-labeled *Torpedo* membrane fragments by carbachol and a series of bischoline ester derivatives. The membrane concentration was 0.1~0.2 mg protein/ml. Excitation and emission wavelength were 280 and 430 nm, respectively. Small volumes of Carb as well as bischoline esters were repeatedly added to the IAS-labeled membranes to a final concentration of 10 μM Carb and of 9.5 μM. bischoline esters. The fluorescence enhancement was monitored upon the agonist binding and fitted to a non-linear simple-binding regression as described in the method. Data were correlated for nonspecific effects by parallel titration of receptor membranes preincubated with α-BuTx. Quantitation yielded values of K_d and F_{max} (maximum fluorescence enhancement in arbitrary units) were: K_d = 0.536μM and F_{max} = 19.15 for Carb (A), K_d = 1.431 μM and F_{max} = 38.35 for succinyldicholine (B), K_d = 0.368 μM and F_{max} =12.51 for azelyldicholine (C).


Fluorescence Enhancement (Arbitrary Units)
2.5
2.0
1.5
1.0
0.5
0.0
0 1 2 3 4 5 6 7 8 9 10
A
Carbachol Concentration (μM)
Fluorescence Enhancement (Arbitrary Units)
1.25
1.00
0.75
0.50
0.25
0.00
0.0 2.5 5.0 7.5 10.0
B
Succinyldicholine Concentration (μM)
Fluorescence Enhancement (Arbitrary Units)
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1.5
1.0
0.5
0.0
0.0 2.5 5.0 7.5 10.0
C
Azelyldicholine Concentration (μM)

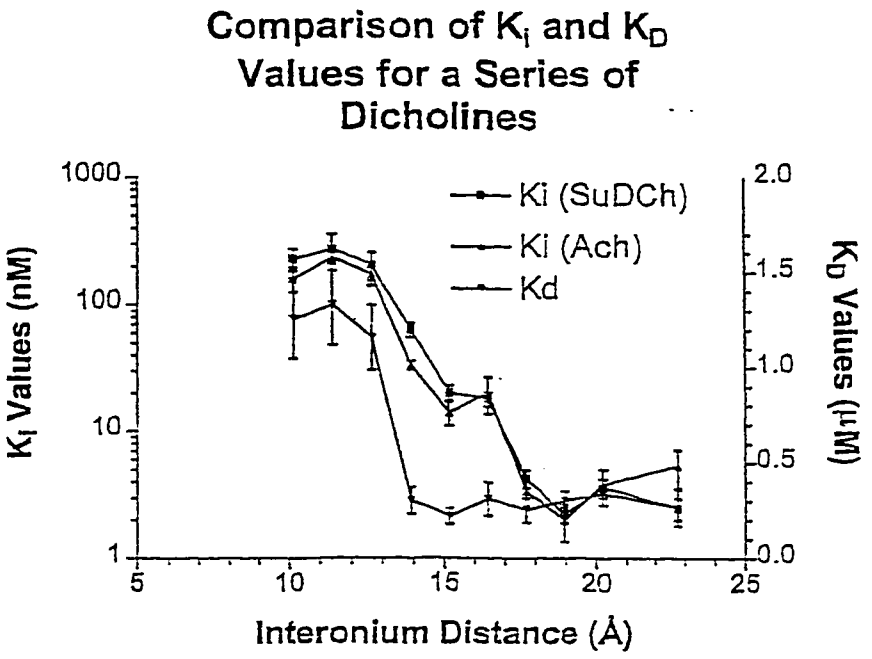


Figure 9. Comparison of affinities of bischoline esters obtained from equilibrium displacement binding assays and equilibrium titration experiments. The K_i and K_d values were plotted versus interonium distance of a series of bischoline esters. There were three levels about the K_is in both displacement of [^{3}H]Ach (▲) and [^{3}H]SdCh (■) by these bischoline ligands, while two levels about K_ds in IAS fluorescence enhanced by these bischoline esters (▼).

between C4/C5 is seen in both studies. Another affinity break between C7/C8 is also observed from equilibrium displacement assays but not in the fluorescence titration experiments.

5. Discussion

The binding of cholinergic agonists to the nAchR promotes transitions of the receptor from the resting state to the activated state and, if the agonist remains bound to the receptor, to the desensitized state. For Ach, the dramatic difference in its affinities for different states is believed to be attributed to these conformational transitions; the activation EC_{50} is estimated to be 100 μM in Torpedo AchR and its equilibrium dissociation constant is about 10 nM (Dunn, 1993; Devillers-Triery *et al.*,1993). Thus there is a four orders of magnitude difference in affinity between the activated and desensitized states. It is well established that the stoichiometry of high-affinity sites is two per receptor at equilibrium.

In our present studies of equilibrium binding of radiolabeled Ach or SdCh to crude *Torpedo* nAchR, the dissociation constants of [^{3}H]Ach and [^{3}H]SdCh were 14.9 nM and 11.4 nM, respectively without any evidence of cooperativity. These results are consistent with previous observations (Dunn and Raftery, 1993, 1997a) and did not support some other reports which suggested different affinities of Ach at equilibrium (Sine *et al.*,1990; Zhang *et al.*, 1995). Moreover, competition experiments in which unlabeled Ach or SdCh were used to displace [^{3}H]Ach or [^{3}H]SdCh showed that they exhibited similar inhibitory dissociation constants as listed in Table 1. These results indicate that Ach and SdCh are likely to be bound to the same sites and their binding is mutually exclusive at equilibrium.

Recently, investigation of the kinetics of Ach, SdCh and a series of bischoline ester ligands led Dunn and Raftery (1997 a, b) to suggest the existence of two subsites within each of the high-affinity sites of the nAchR molecule.. In automated dissociation assays, the dissociation of [^{3}H]Ach from the high-affinity sites was accelerated in a concentration-

dependent manner in the presence of unlabeled Ach, Carb and SdCh. [^{3}H]SdCh dissociation, however, was not significantly enhanced by the presence of unlabeled ligands. In order to interpret this observation, the authors suggested that there may exist two subsites and only one is occupied by the mono-quaternary ligand but two are cross-linked by the bischoline SdCh at equilibrium (see Figure 3).

In addition to the above evidence, other observations have shown that there may exist two subsites for binding of antagonists and neurotoxins to the nAchR. For example, a flexible bis-quaternary antagonist [^{3}H]bis(3-azidopyridium)-1,10-decane perchlorate (DAPA), was shown to photolabel all four subunits at equilibrium. However, the labeling of the β, γ and δ subunits was significantly diminished after saturating the Cys-192/193 residues by covalently labeling with BrAch whereas the labeling of the α-subunit was not significantly affected, implying that there are two attachment sites for cholinergic ligands on the α-subunits (Tine and Raftery, 1993). Fu and Sine (1994) demonstrated that dimethyl-*d*-tubocurarine (DMT), a rigid bischoline antagonist, bridges the α-γ subunit interface fitting into a pocket bound by α-Tyr-198 and γ-Tyr-117. α-BuTx was proposed to bind to two subsites in the binding domain of α-subunit: the proline subsite consisting of Pro-194 and 197 is critical for α-BuTx binding, and the aromatic subsite containing Tyr-187 and 189 determines the extent of α-BuTx binding (Kachalsky *et al.*, 1995).

The two subsites were further characterized by studying the association kinetics of this series of bischoline ligands to IAS-labeled nAchR. It was reported (Dunn and Raftery, 1997b) that the ligands with 4 methylene groups or less (C6 and shorter compounds) displayed a desensitization process similar to Carb while those with 5 methylene groups or

more (C7 and longer compounds) showed SdCh-like kinetics; thus, there was a break between C6/C7 (AddCh and PidCh) in their association kinetics. Since these compounds have two choline moieties separated by various methylene groups, this intrinsic character could be utilized to understand the nature of their binding sites or subsites. This IAS kinetic study demonstrated that the distance between the two subsites may be approximately determined by the length of C7. By measuring from Tl^+-flux responses induced by these bischoline ligands, a break of their activation constants was obtained between C5/C6 but not C6/C7 (Kawai, 1998). These results indicated that the distance between the two subsites may differ in the resting and activation states.

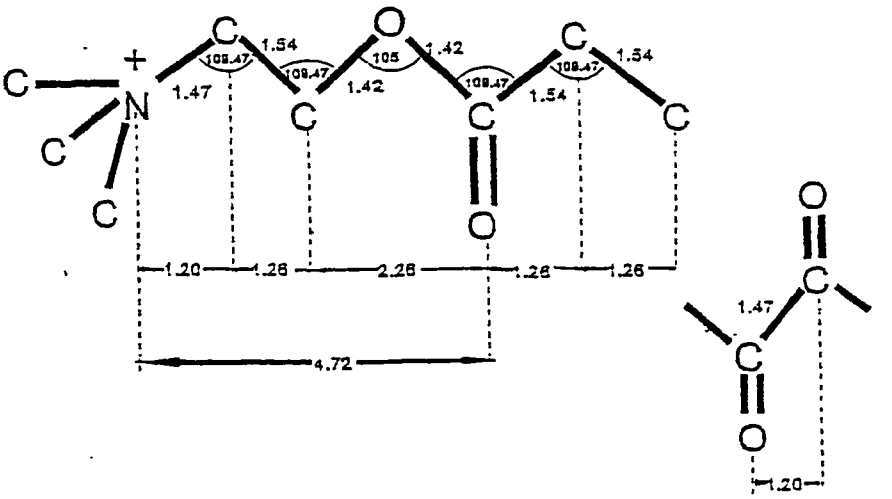
In the present studies, these bis-quaternary ligands were used for further investigation of their abilities to displace radiolabeled Ach and SdCh, and for measurement of dissociation constants for these ligands by measuring IAS-fluorescence changes. The affinities and the trend of affinity change of these bischolines obtained in equilibrium displacement assays using [^{3}H]Ach and [^{3}H]SdCh were similar. The affinities were divided into three groups: C2-C4 (K_i~ 100-200 nM), C5-C7 (K_i~10-30 nM) and C8-C12 (2-5 nM). Two breaks between C4/C5 and C7/C8 were observed. All these bischolines were found to be able to completely displace the radiolabeled ligands. This indicated that the binding of these bischolines and the radioligands is mutually exclusive at equilibrium.

Unlike the equilibrium displacement assays, the K_d values of these bischolines were divided into two groups from the equilibrium IAS-fluorescence titration experiments: C2-C4 (K_d~ 1.2 μM) and C5-C12 (K_d~ 0.3 μM) with one break between C4/C5. No break between C7/C8 was obtained.

In their recent two-subsite model, Dunn and Raftery (1997 a, b) suggested that each of the two high-affinity sites contains two mutually exclusive subsites (e.g. subsite A and subsite B), one subsite being a primary site and the other being a secondary site. It was suggested that if Ach originally occupies the primary site A, a second Ach molecule may bind to the other site B only at higher concentration (EC_{50}=2.1 μM) and this accelerates the Ach dissociation from the primary site; thus only one site, either A or B, could be occupied at equilibrium. In contrast, SdCh, being a large bis-quaternary molecule, may physically bridge both primary and secondary subsites; this crosslink prevents another SdCh molecule from binding. Our results provided further evidence to support this two-subsite model in that it may be predicted that a ligand binds to the receptor with higher affinity when it is able to physically bridge the two subsites. A series of bis-quaternary ligands were shown to display three groups of K_is from displacement study and two groups of K_ds from fluorescence titration study of IAS-labeled receptor. These bischolines contain two quaternary ammonium groups, both of which are assumed to be able to bind to distinct subsites on the receptor. When the bischoline ligand is too short to cross-link the two subsites, it is expected to behave like Ach (mono-quaternary ligand) and show lower affinity to displace radioligands. In our studies, the affinities of C2-C4 are the lowest indicating that these ligands may only bind to subsite A or subsite B, but not both which is similar to Ach. It was also noted that the affinities of C2-C4 are approximately ten-fold lower than that of Ach. This may be because these compounds, larger than Ach, have more steric hindrance in their binding. On the other hand, C5-C12 showed higher affinities, suggesting that they may be long enough to bridge the two subsites.

Both studies of equilibrium displacement and fluorescence titration of IAS-labeled receptor showed an apparent break between C4/C5 for binding to the receptor at equilibrium. In contrast to our results, breaks between C6/C7 from studies of the kinetics of binding to IAS-labeled receptor (Dunn and Raftery, 1997b) and between C5/C6 from measurements of the ion flux response (Kawai, 1998) were observed. This suggests that there may be differences in the binding of these ligands to the resting, activated and desensitized states of the receptor. The shift in the breaks of the affinities from C5/C6 for activation to C4/C5 for desensitization states may indicate shortening of the two subsites for the positively charged quaternary amines due to the conformational changes leading to desensitization. Moreover, these differences of affinity breaks may also reflect the presence of distinct binding sites of activation and desensitization, as previously reported (Dunn and Raftery, 1982a,b; Raftery *et al.*, 1983; Dunn *et al.*, 1983; Dunn and Raftery, 1993).

We cannot know the exact distance between the two subsites from our studies because of the high flexibility of these bischolines and the uncertain conformation of the bound ligand. We can, however, approximately estimate this distance based on the hypothesis that the two subsites must be closer together than the extended form of the shortest ligand that can cross-link them. The distance between the quaternary amines in this bischoline series can be estimated as follow: N-C (1.20 Å), C-C (1.26 Å) and the ether C-O-C (2.26 Å) as shown in Figure 10. This calculation is based on a molecular structure assuming that the carbon bond angle is 109.47° and that of oxygen being 105° rather than molecular extension as N-C (1.47 Å), C-C (1.54 Å) and C-O (1.43 Å) (Fieser and Fieser, 1957). Considering that the main break is between C4/C5 in our studies, the distance



Acid	Interonium Distance (Å)
Oxalic	10.12
Malonic	11.38
Succinic	12.64
Glutaric	13.90
Adipic	15.16
Pimelic	16.42
Suberic	17.68
Azelaic	18.94
Sebacic	20.20
Dodecanedioic	22.72

Figure 10. Interonium distance of a series of bischoline ligands. Published bond lengths were used as follows: N-C (1.47 Å), C-C (1.54 Å) and C-O (1.43 Å) (Fieser and Fieser, 1957). The distance calculated between the projections of constituent atoms on the axis of the fully extended molecule were determined by trigonometry, assuming the carbon bond angle to be 109.47° and that of oxygen to be 105°. Therefore, the projected bond lengths were: N-C (1.20 Å), C-C (1.26 Å) and C-O-C (2.26 Å). From these calculations, the dimensional lengths were determined for the series of bischoline esters thereafter.

between the two subsites at equilibrium may therefore be predicted to be between 12.64 Å of C4 (SudCh) and 13.90 Å of C5 (GldCh). This distance is slightly longer than that estimated by Fu and Sine (1994) who predicted that DMT bridged the two attachment residues at the α-γ interface with a distance of 10.8 Å. The rigidity of DMT and sharing of the common binding domain with distinct but overlapping subregions by agonist and antagonist may account for this distance difference.

Some studies report that the Ach binding sites are situated approximately 30 Å above the plane of the membrane (Unwin, 1993; Valenzuela *et al.*, 1994); thus it is quite rational to speculate that the binding sites of nAchR for its cholinergic ligands could be located at either the outer or the inner perimeter of the cylindrical vestibule. Kistler *et al.* (1982) reported that α-BuTx, the molecular weight of which is 8,000, binds to the outer perimeter. However, our present studies suggest that these smaller bischoline ligands are more likely to bind at the inner than at the outer perimeter. Since there is accumulating evidence to suggest that the two high-affinity sites of nAchR are formed at the interfaces of α-γ and α-δ subunits (Pedersen and Cohen, 1990; Middleton and Cohen, 1991; Dunn *et al.*,1993), the two primary subsites observed in this study may be located at these subunit interfaces. Figure 11 shows the putative binding subsites at the subunit interfaces. The high affinity sites located at the α-γ and α-δ subunit interfaces are shown in green. Two circles of radius 14 Å (the chain length of C5) intersect the interfaces between α-β, β-δ and α-γ, shown in red, on its inner perimeter. These sites are proposed to be the secondary subsites (Dunn and Raftery, 1997a,b) or even the distinct low affinity sites previously reported (Dunn and Raftery, 1982 a,b; Raftery *et al.*, 1984, 1985). It is therefore possible for an extended C5

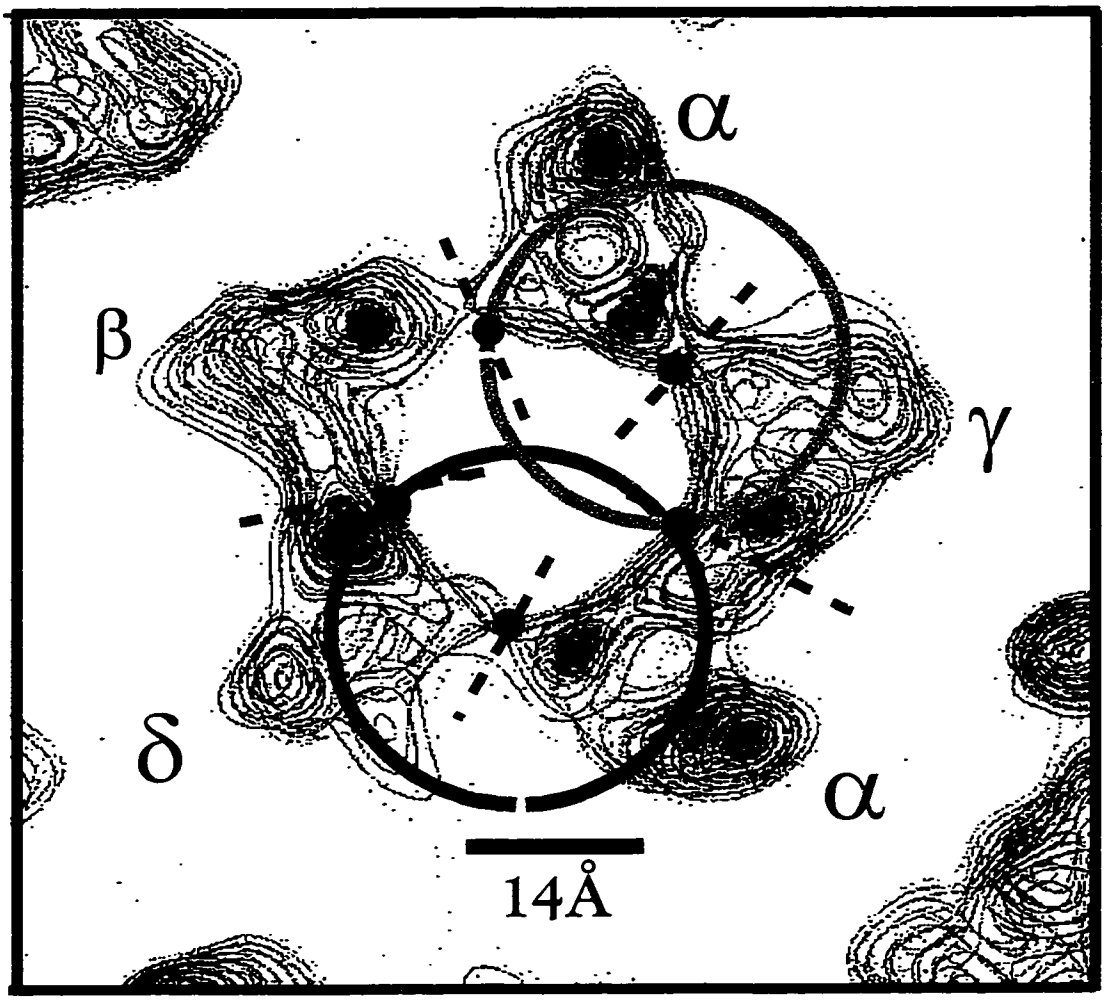


Figure 11. Putative agonist binding sites at the subunit interfaces at a resolution of 7.5Å. This 12Å thick section (modified from Unwin, 1996) lies 30Å above the membrane bilayer and contains putative high affinity binding sites (green points) at the α-γ and α-δ interfaces (Pedersen and Cohen, 1990; Sine and Claudio, 1991). Two circles of radius 14Å, centred on the high affinity sites, intersect the two adjacent interfaces at the inner perimeter of the channel opening (red points). These sites may be the secondary subsites suggested by Dunn and Raftery (1997a,b) or even distinct low affinity sites (Raftery *et al.*, 1984, 1985). An extended C5 molecule is the shortest bischoline in the series that can span the high affinity sites and the secondary or low affinity sites.

molecule (or longer analogs) to span the putative binding sites if indeed they are located on the inner perimeter.

The composition of the two subsites is still unclear. However, many studies showed that each high-affinity binding site is composed of multiple attachments of amino acid residues forming a binding pocket or gorge to interact with its agonists, antagonists and neurotoxins (Abramson *et al.*, 1989; Cohen *et al.*,1991; Galzi *et al.*, 1990,1991a,b; Tomaselli *et al.*, 1991; O'Leary and White, 1992; O'Leary *et al.*, 1994; Sine *et al.*, 1994; Chen *et al.*, 1995; Czajkowski and Karlin, 1995; Martin and Karlin, 1997). Therefore, the two subsites may be composed of the aromatic amino acids to stabilize the quaternary ammonium groups of agonist and antagonist through cation-π interaction (Dougherty and Stauffer, 1990). In addition, there are some acidic side chains could also serve a presumed long-range coulombic interaction (Stauffer and Karlin, 1994; Sugiyama *et al.*, 1996; Tsigelny *et al.*, 1997) in stabilizing the complex in one or both of the subsites. These bischolines may interact with distinct amino acid residues than each other or the same residues but with different affinities.

In the fluorescence experiments, a clear break between C4/C5 was observed with no evidence for any group displaying intermediate affinity. It was reported previously that IAS-labeling did not affect the binding of the monoquaternary agonists, e.g. Carb and Ach, to their high affinity sites (Dunn *et al.*,1980); our present study supports this observation in that IAS-labeling did not alter either the dissociation constant or the number of receptor binding sites. However, IAS-labeled receptor may have, to some extent, interfered with the binding of the larger bisquaternary ligands. This could explain the absence of the affinity break

between C7/C8 seen in the fluorescence experiments. It was found that the affinities of all ligands of this bischoline series from fluorescence study were lower than those from equilibrium displacement assays, e.g. there was a 70~80-fold affinity difference between C2 and C8 from displacement binding and this affinity difference diminished to 4~5-fold from the fluorescence study. Although it was indicated previously (Dunn *et al.*, 1980; Blanchard *et al.*, 1982), and also in the present results, that IAS labeling did not affect the binding of the monoquaternary agonist, e.g. Carb, it is possible that the IAS labeling and/or the reduction of disulfide bond between α-Cys-192/193 by DTT may affect the binding of these longer bisquaternary ligands.

In summary, the present studies of equilibrium displacement and fluorescence titration experiments provide further evidence of the existence of the two subsites within each high affinity sites of the nAch receptor. They are possibly located at the inner perimeter of the subunit cylinder, approximately 30Å above the membrane bilayer and the distance between them being approximately 13.90 Å at equilibrium (Figure 11).

Chapter 2

Characterization of Agonist-Induced Desensitization of the Nicotinic Acetylcholine Receptor

1. Introduction

1. 1. Semirigid Cholinergic Agonist

Arecolone methiodide (Arecolone-MeI) is a semirigid and potent cholinergic agonist (Figure 12; Spivak *et al.*, 1983). In studies of the binding of arecolone-MeI to the *Torpedo* nAchR by measuring its ability to inhibit the rate of ^{125}I-α-BuTx binding, these authors demonstrated that it was 4-fold more potent than Carb, and half as potent as Ach (Spivak *et al.*,1989). Kawai (1998) has recently directly measured the binding of [^{3}H]arecolone MeI and showed that, at equilibrium, it binds three-fold less tightly than Ach. However, in Tl^+ flux measurements, this ligand is 2~3-fold more potent than Ach when activating the receptor. To further investigate the two-subsite model, rapid dissociation studies were carried out (Kawai, 1998). Like other agonists, arecolone-MeI was shown to accelerate [^{3}H]Ach dissociation from the high affinity binding sites. When used to accelerate [^{3}H]SdCh dissociation, arecolone-MeI was found to be 15 times more potent than Ach. These results led Kawai to suggest that this semirigid agonist, like other monoquaternary ligands, may show preference in occupying one of the two subsites on the receptor postulated previously by Dunn and Raftery (1997a, b).

In a preliminary fluorescence titration study, arecolone-MeI was observed to induce IANBD fluorescence enhancement with an apparent K_d = 30 μM which is similar to EC_{50} (36.7 μM; Kawai, 1998) for receptor activation. However, no fluorescence changes were found when arecolone-MeI was titrated to IAS-labeled nAchR. This suggested that arecolone-MeI may bind to the distinct low-affinity binding sites to activate the receptor, but not to the sites that promote desensitization (Dunn and Raftery, personal communication).

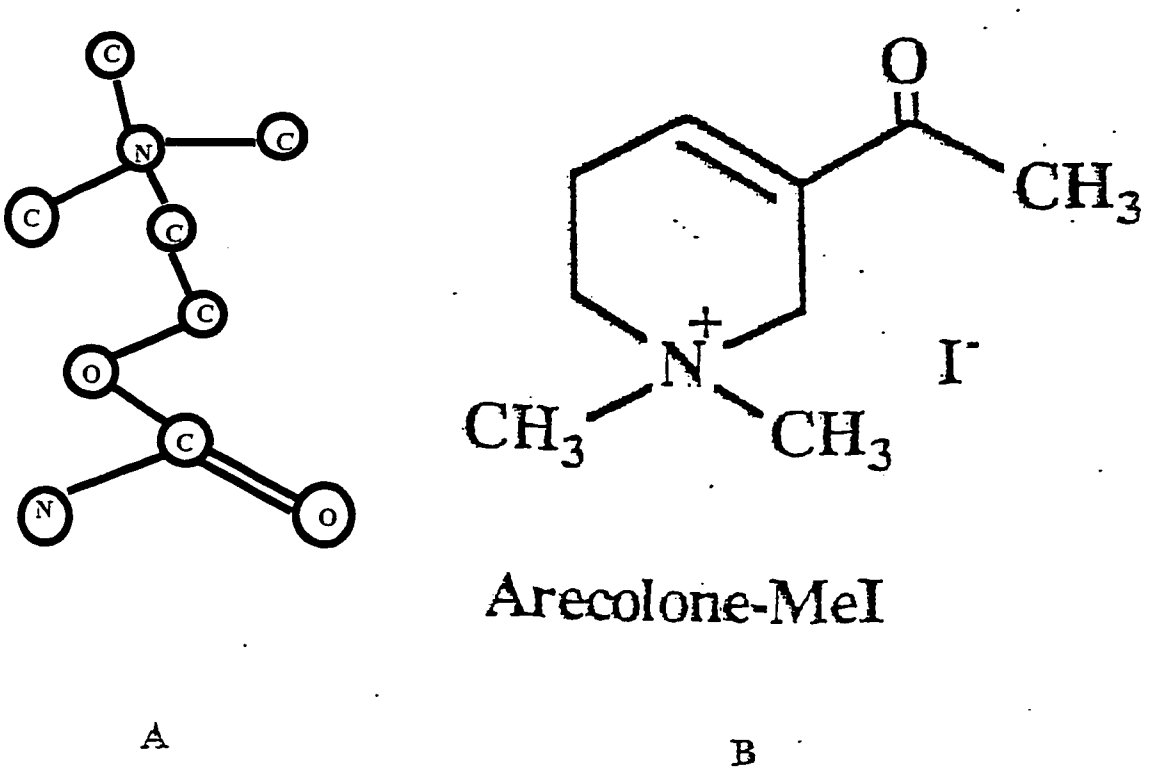
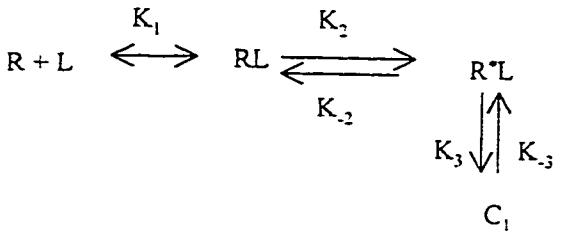
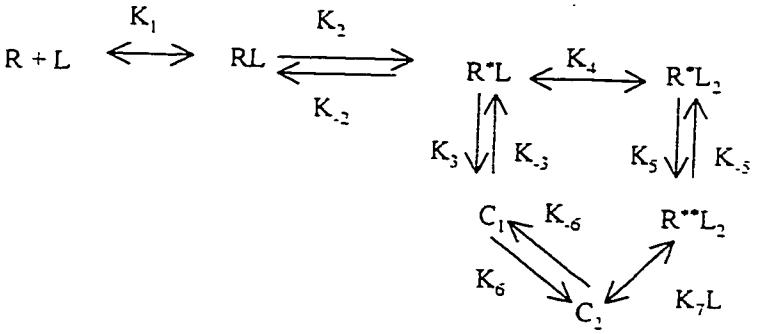


Figure 12. Structure of carbachol and arecolone-MeI. (A) Carb is a flexible agonist that could adjust its structure to fit the binding pocket. On the other hand, arecolone-MeI (B) is rather rigid quaternary amine, with its acetyl group attached at the position 3 of the dihydropyridine moiety to form a meta configuration.

In their recent studies of kinetics of SdCh binding monitored by IAS fluorescence, Dunn and Raftery (1997 b) postulated a kinetic scheme that two sequential conformational changes, a fast and a slow phases, followed the initial complex and the rates of these two transitions are concentration independent.



However, when the kinetics of Ach was analyzed, the slow phase was evident only at low ligand concentrations and replaced by an intermediate one when higher concentrations (> 20 μM) were used. Therefore, a distinct kinetic scheme was suggested for Ach: at low ligand concentrations, the reaction proceeds by the slow pathway toward desensitization state C_1, at higher ligand concentrations, a second molecule is capable of binding to the other subsite with lower affinity, and this occupancy induces further conformational changes, $R^*L \rightarrow R^*L_2$, toward desensitization state C_2.



To understand the structure-activity relationship at the molecular level, one approach is to identify a rigid and potent agonist for further characterization of the binding sites. As shown in Figure 12, Carb is a flexible ligand, i.e. it can, like Ach, induce the conformational changes of the receptor upon its binding. On the other hand, a dihydropyridine moiety in arecolone-MeI is expected to resist such conformational changes. When bound to its binding sites, this potent property can be investigated by comparing channel gating parameters evoked by arecolone-MeI with those associated with a more conventional agonist.

1. 2. Isolated Patch-Clamp Technique

Patch-clamp techniques were first introduced by Neher and Sakmann (Neher and Sakmann, 1976) and improved several years later (Hamill *et al.*, 1981). The essential character of the patch clamp is to seal a small membrane area (perhaps 1-2 μm^2) with the tip of an absolutely clean and smooth glass pipette and isolate this membrane patch from the rest of the cell membrane. By doing this, the electrical noise level is significantly reduced and the currents flowing through the single channels in the membrane patch can be measured. This allows the properties of these channels to be investigated. Combined with negative pressure, a high resistance seal between the electrode tip and the cell membrane can be obtained. This tight gigaohm seal has two advantages: the ability to measure a small detectable current and a mechanically maintained stable seal (Sakmann, 1992; Neher and Sakamnn, 1992). From their studies of ligand-gated ion channels, at least two conductance states have been characterized: a closed channel in the absence of bound agonist and a fully opened channel when the binding sites are occupied. In the activated state of the receptor, the channel does not remain open throughout, but rather fluctuates between opened and

closed states many times until the agonist either dissociates from the receptor or the receptor desensitizes. The amplitude of the single-channel current is an index of ion flux through the channel, mainly Na^+ and K^+ ions; and the length of the open time is a function of the ligand itself and its interaction with the binding sites. Inside-out single-channel recording, combined with outside-out and whole cell recordings, are three commonly used patch-clamp techniques. In the former techniques, after forming the tight gigaohm seal, the electrode is quickly withdrawn from the cell membrane so as to form an excised inside-out patch with the membrane sealing the electrode orifice and the electrode lumen forming a closed system (Hamill *et al.*, 1981). The extracellular side of the membrane faces the electrode lumen and the intracellular side faces the bathing solution.

2. Specific Objective

To gain more insight into the mechanisms underlying Ach receptor channel gating, two agonists, Carb and arecolone-MeI, have been used in the eletrophysiological study. Carb is a flexible molecule capable of adopting a change of conformation. In contrast to Carb, the rigidity of arecolone-MeI may restrict such a conformational change of receptor and itself. In the nAchR reconstituted in giant liposomes, we can compare the single-channel conductance, open time duration and onset of the desensitization induced by either Carb or arecolone-MeI. In this way the model for receptor activation proposed by Dunn and Raftery (1997 b) (and see above) can be challenged by ligands that impose differential restraints on receptor conformational changes.

3. Methods

3. 1. Single-Channel Recording of Ion Channel Reconstituted in Giant Liposome

3. 1. 1. Preparation of Giant Liposome

The procedure used in our studies was modified from that previously described (Criado and Keller, 1987; Keller *et al.*, 1988; Riquelme *et al.*, 1990). L-α-lecithin (L-α-phosphatidylcholine) and CHAPS were suspended and vortexed in distilled water to make 10 ml mixture. The final concentration was 50 mg/ml lecithin and 1.5% (w/v) CHAPS. This mixture was kept at 4°C for overnight dissolution, divided into 500 μl aliquots and stored at -86°C. Crude membranes from *Torpedo* electric organ were prepared as described above except that the final pellet was suspended in dialysis buffer (10 mM Hepes, pH 7.4, 100 NaCl). Routinely, 400 μl aliquots of the mixture of lipid and detergent, 200 μg aliquots of membrane fragments and dialysis buffer were mixed to a final volume of 1 ml. The mixture was then incubated for 1 hr at 0°C. Lipid vesicles were formed on removal of the detergent by exhaustive dialysis against dialysis buffer for 24 hr. The mixture was diluted with dialysis buffer and then centrifuged for 1 hr at 100,000g. The resulting pellet was resuspended at 4°C in 75 μl of dialysis buffer containing 5% (v/v) ethylene glycol by forcing the suspension through the needle of a small syringe until an apparently homogeneous suspension was produced. The suspension was deposited as small droplets (~ 4 μl) on 3.5-cm Petri dishes and submitted to partial dehydration for 3 hr at 4°C in a desiccator containing anhydrous $CaCl_2$. The samples were rehydrated by covering them with 4 μl rehydration solution (equal volume of dialysis buffer and distilled water) at 4°C overnight in large, closed

petri dishes containing a wet towel paper on the bottom. The rehydrated drops were rinsed off with experimental buffer (4 mM Hepes, pH 7.4, 50 mM NaCl, 0.1 mM $CaCl_2$) and centrifuged at 1,000 rpm for 10 min. The pellet was collected by resuspension in 1 ml experimental buffer and stored at 4°C for later use.

3. 1. 2. Patch-Clamp Recording of Ion Channel

Aliquots (75-100 µl) of giant liposomes were pipetted into 3.5-cm petri dishes, incubated with 1 ml experimental buffer for 5-10 min allowing the liposomes to deposit on the bottom of the dish.

Single-channel recordings were obtained by using patch-clamp techniques as described by Hamill *et al.* (1981). The experimental buffer was used both in the bath medium and inside the electrode (referred to as "symmetrical" conditions). Patch electrodes were pulled from thin-walled bososilicate glass capillaries (VWR Scientific, West Chester, PA) by a two-stage pulling procedure with a vertical electrode puller (L/M-3p-A, Adams & List Associates Ltd, Westbury, NY), and subsequently coated with Sylgard®, starting 50 µm from the tip. The tip of the electrode was then fire-polished on a microforge. The electrode resistance was 5-25 MΩ when filled with the inside-out solution. Unless mentioned, all the single-channel recordings were performed at room temperature (23°C).

Giga seals (10-20 GΩ) were obtained on giant liposomes with electrodes containing equipotent concentrations of Carb and arecolone-MeI (5 µM Carb and 2 µM arecolone-MeI, Spivak *et al.*,1989) by apposing the electrode tip to the exposed liposome surface with the help of a manipulator and applying gentle suction to the electrode interior. After the

electrode was sealed, careful withdrawal of the electrode from the liposome surface and, if necessary, quickly passing the tip through the air/water interface resulted in an excised patch in the inside-out configuration. An Axopatch-1D patch-clamp amplifier (Axon Instruments Inc. Foster City, CA) was used, at a gain of 100 mV/pA and filter setting of 5 KHz. The holding potential was +65 mV, applied to the interior of the patch electrode, and the bath electrode was maintained at virtual ground. The single-channel currents were monitored simultaneously on an oscilloscope (5113, Tektronix Inc. Beaverton, OR) and stored on a magnetic tape recorder (PI-6200, Precision Instrument Co. Palo Alto, CA).

3. 2. Data Analysis

All recorded signals were filtered at 0.5 KHz by a LPF-100 low-pass filter, digitized by a digital-analog converter (TL-1 DMA Interface, Axon Instrument Inc. CA) and then analyzed on a PC computer with pClamp software package (Version 5.5, Axon Instrument Inc. CA). Only single-channel events with durations longer than 200 μs were used for the amplitude analysis. Routinely, open-channel events were plotted by fitting a Gaussian histogram to the current amplitude distribution (number of events vs current amplitude with bin width 0.1 pA) and the mean amplitude values were obtained thereafter. The time constant (τ) for the open-channel state was determined by fitting of an exponential to the frequency plot with a bin width 0.2 ms.

4. Results

Inward currents occurred immediately when a holding potential of +65 mV was applied in the presence of either agonist and spontaneously terminated in a similar time range (Figure 13). Carb-induced currents were observable for periods ranging from 1 to 34 min after which the currents disappeared. This disappearance was considered as receptor desensitization. The onset of desensitization in the presence of Carb was variable. From 30 patches, 80% had a duration of single channel current activity $\leq$ 10 min. Similarly, the duration during which arecolone-MeI-induced current was observable ranged from 2 to 26 min and 66.6% had period of observable activity $\leq$ 10 min (Figure 14). Therefore, desensitization induced by Carb was faster than that by arecolone-MeI. In the presence of 5 μM Carb, only 13.3% of the patches (30 out of 225 recorded patches) exhibited detectable responses. Likewise, 15.5% of the patches (15 out of 97) investigated in presence of 2 μM arecolone-MeI exhibited currents.

There were two conductance levels produced by each agonist. Figure 15 A shows the histogram of current amplitude plotted against frequency in the presence of Carb. Two clear peaks of current amplitudes were observed and the mean values were determined to be 2.2 ± 0.2 pA (mean ± S.D.) and 3.6 ± 0.1 pA, respectively. The point open channel conductances, estimated from the mean amplitude values over holding potentials, were 34 pS and 56 pS respectively. The numbers of events recorded at the higher conductance level was slightly more than those at the lower level, 2150 out of 3763 recorded events (57.1%) were at the higher conductance level and 42.9% (1613 out of 3763 recorded events) at lower level (Figure 15 B). The time constant (τ) of open-channel state was found to be 2.83 ms and

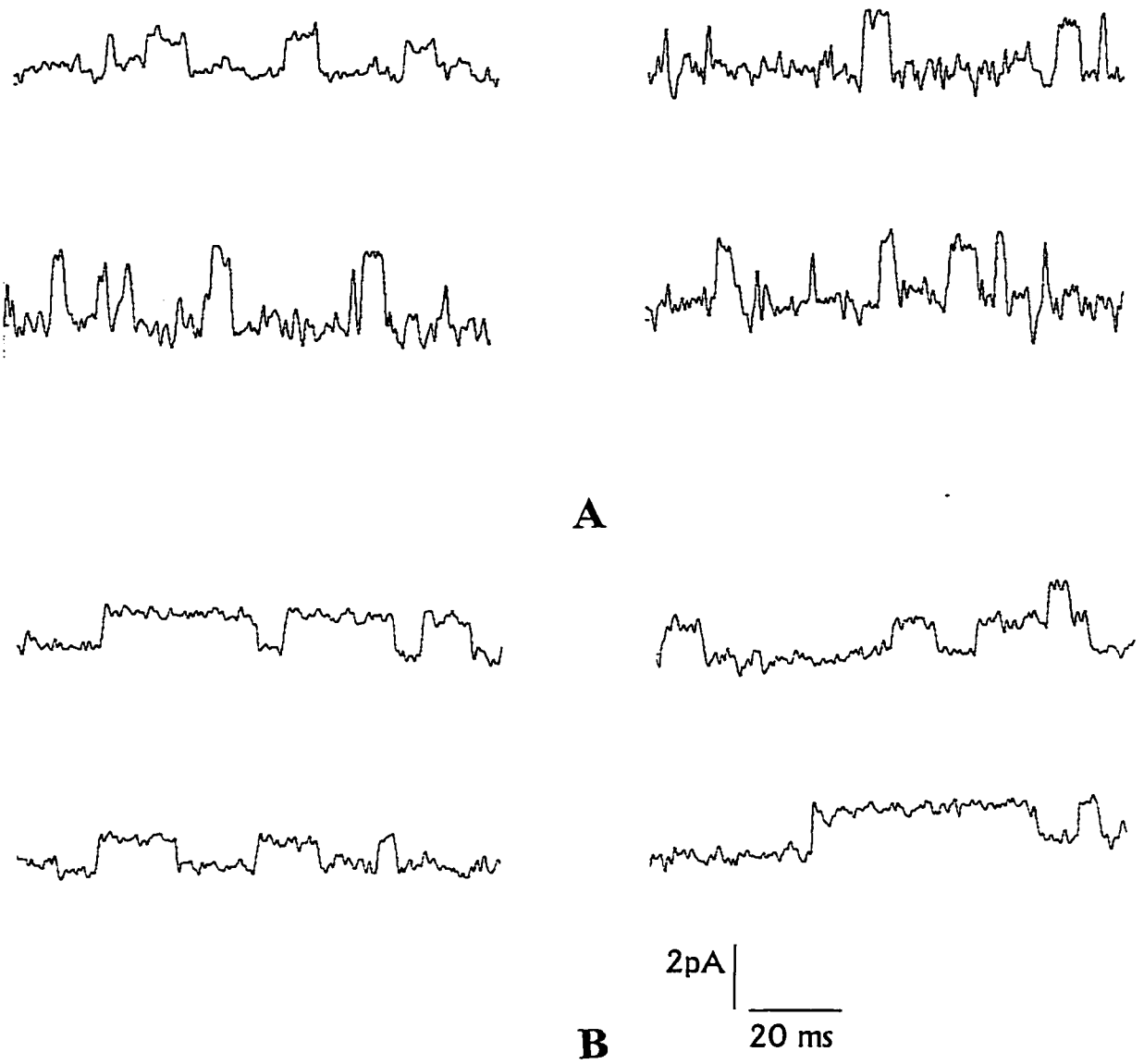


Figure 13. Main conductance and a subconductance state of the inside-out single-channel recording of the reconstituted *Torpedo* nAchR. (A) carbachol-activated single-channel currents. Pipette holding potential, +65 mV; 5 μM Carb; the conductances were 34 and 56 pS, respectively. (B) arecolone-MeI-activated single-channel currents. Pipette holding potential, +65 mV; 2 μM arecolone-MeI; the conductances were 33 and 58 pS, respectively. A upward deflection is defined as channel opening events.

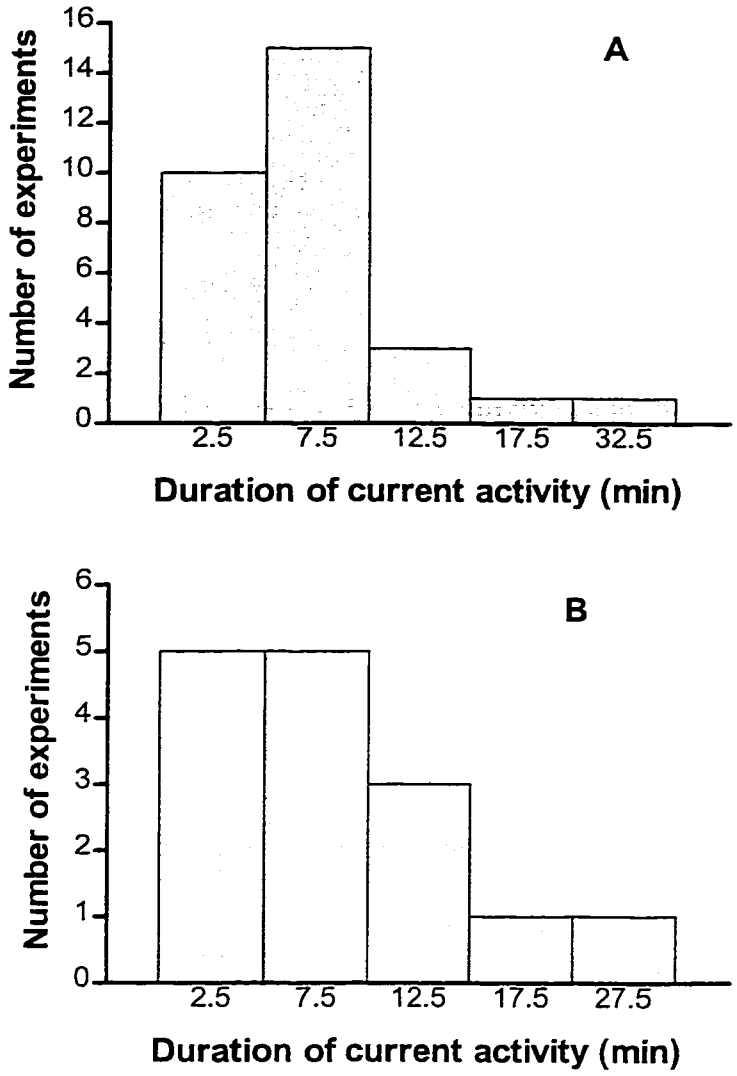


Figure 14. The onset time duration of receptor desensitization induced by either 5 μM carbachol (A) or 2 μM arecolone-MeI (B). The termination of single-channel currents is considered to be the receptor desensitization. The onset time of absence of current by either ligand occurs at the same time duration. For Carb, it is ranged from 1-34 min with 80% patches having a duration of current activity less than 10 min. For arecolone-MeI, the time range is 2-26 min with 66.6% patches having period of current activity less than 10 min.

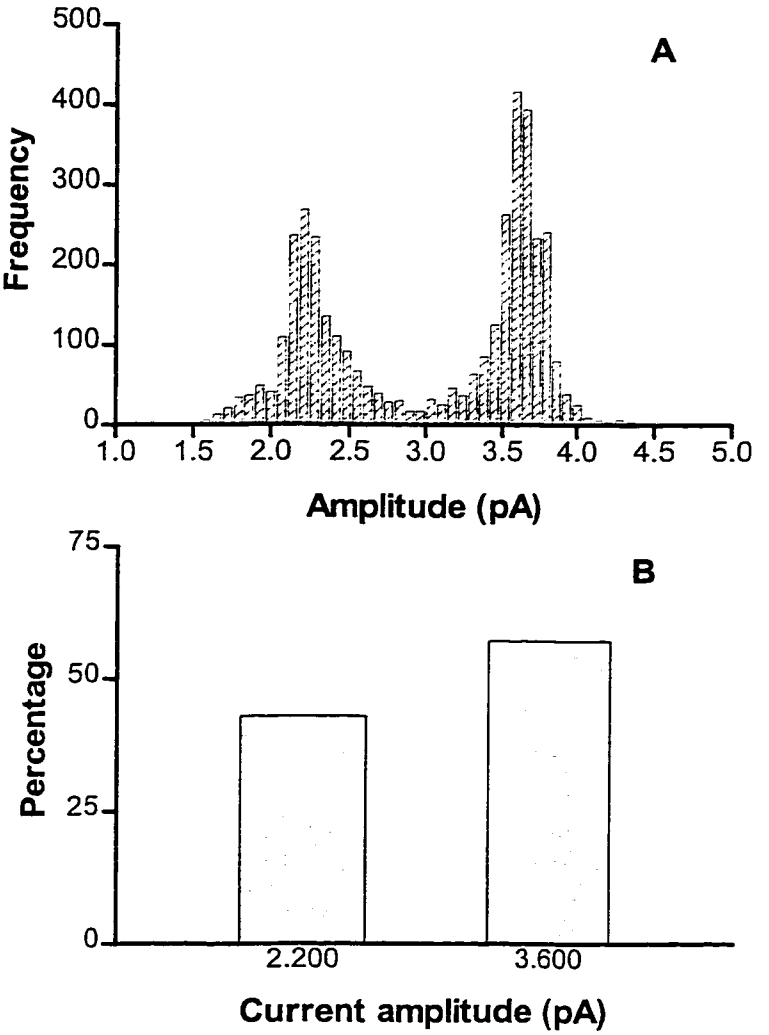


Figure 15. Distribution of carbachol-activated single-channel currents recorded from *Torpedo* nAchR reconstituted into giant liposomes. (A) Histogram is plotted to fit Gaussian distribution as relative frequency of occurrence of a given current level. Two clear peaks are obtained, the first one with a mean value at 2.2 pA and the second with a peak at 3.6 pA. The open conductances were estimated thereafter as one being 34 pS and the other being 56 pS. (B) The percentage of main and subconductance states. 42.9% recorded open event is located at the lower conductance level while 57.1% at high level. The holding potential was +65 mV

the half-life time was 1.96 ms as illustrated in Figure 17 A.

The representative histograms of current amplitude and open-time of arecolone-MeI-induced currents are shown in Figure 16 A. Like the Carb-induced current, two peaks of current amplitude against frequency were observed at 2.2 ± 0.1 pA and 3.8 ± 0.2 pA as the mean values, and thus the calculated point conductances were 33 pS and 58 pS, respectively. It appeared that the conductances generated by these two agonists were close, which is consistent with the concept that the single-channel conductances are independent of the nature of the agonist (Tank *et al.*, 1983; Labarca *et al.*, 1984). However, the open-channel events of arecolone-MeI preferentially occurred at the lower conductance level with 3660 out of 3792 recorded events (96.5%) being at the lower conductance level and only 132 events (3.6%) at the higher level as shown in Figure 16 B. The time constant of the open channel state for arecolone-MeI was determined to be 3.81 ms and the half-life time was 2.64 ms as shown in Figure 17 B, slightly but not significantly longer than those for Carb.

As illustrated in Figure 13, Carb-induced ion-channel currents normally opened to either one conductance state or the other, and rarely exhibited transitions from one conductance state to the other, where they occurred transitions from low to high and high to low were approximately equally frequent. On the other hand, most arecolone-MeI-generated currents appeared to stay at the subconductance state once the channel opened. In very few cases, the full conductance currents were found in the middle of the subconductance state, i.e. the channel initially opened to the subconductance state and would remain at this state until the channel closed or briefly reached the fully opened state and either went back to the subconductance state quickly or closed the channel as shown in Figure 13 B. In proportion

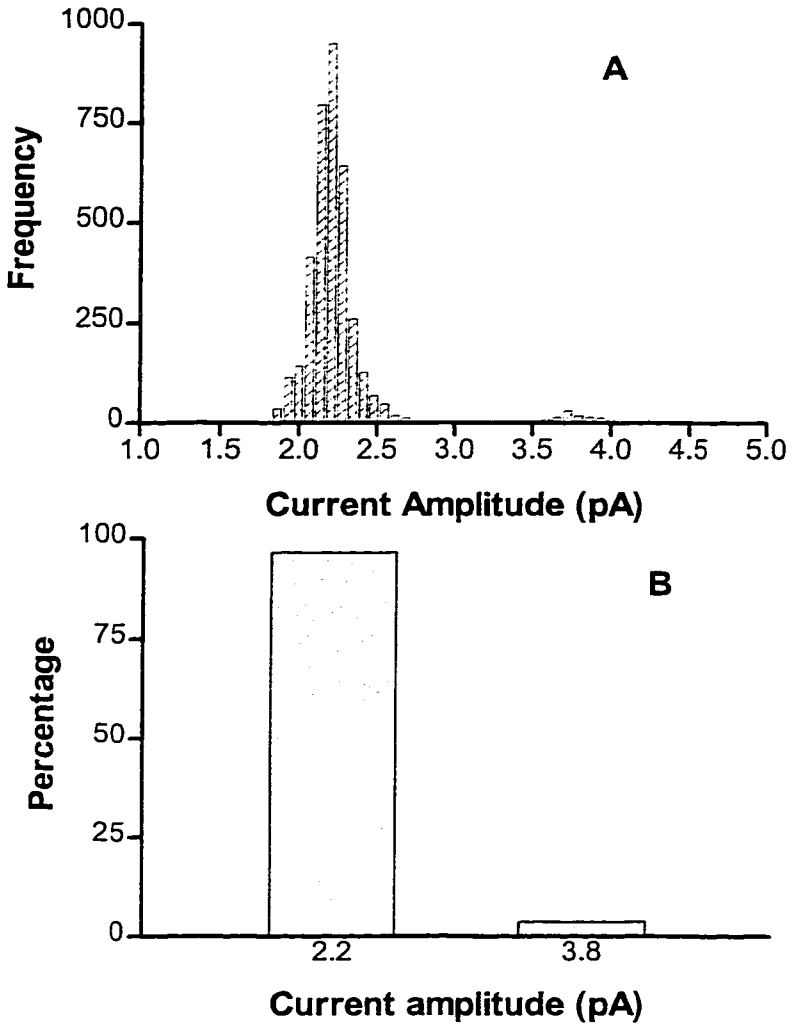


Figure 16. Distribution of arecolone-MeI-induced single-channel currents. (A) Histogram of single-channel currents evoked by 2 μM arecolone-MeI. Holding potential +65mV. Two different current levels plotted against occurrence frequency are obtained. The curves are Gaussian fits to the current amplitude which have mean values of 2.2 pA and 3.8 pA. The calculated conductances are 33 and 58 pS, respectively. (B) Percentage of two conductance levels. The open-channel events of arecolone-MeI preferentially favor the lower conductance with 96.5% recorded events being at this level, while only 3.6% events being at the higher conductance level.

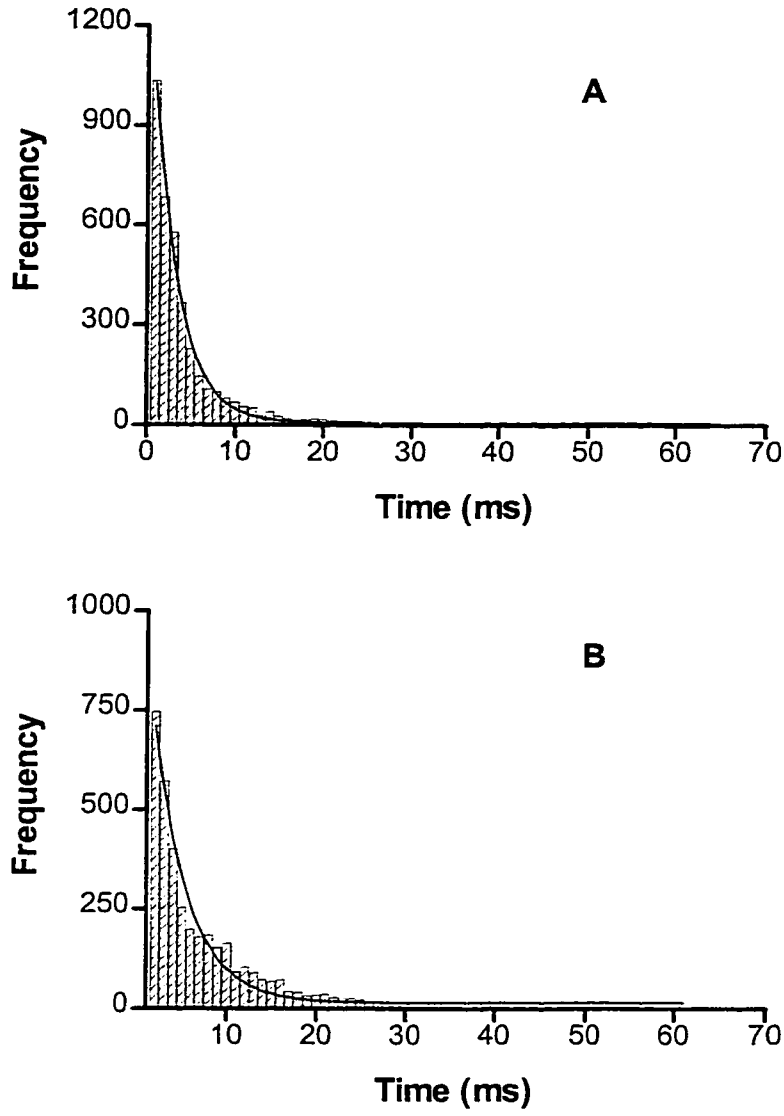


Figure 17. Histograms of open times of single-channel currents induced by either carbachol or arecolone-MeI. Both histograms of open channel lifetime fit in single exponential distribution with time in the frequency of channel opening events. Pipette holding potential is +65 mV. The time constant of 5 μM Carb is 2.83 msec (A) and 3.81 msec for 2 μM arecolone-MeI (B).

of Carb-generated events recorded at the lower conductance state was 42.9%, the proportion of arecolone-MeI-generated events recorded at the lower conductance, however, was 96.5%.

5. Discussion

In earlier studies, native *Torpedo* receptors were found to be very difficult to investigate in single-channel recording experiments because of the fragility of the membrane lipids and the extremely high density of the receptors. Using artificial membrane phospholipids, reconstitution of nAchR and measurements of the excised inside-out patch-clamp technique allows detailed analysis of the functional and pharmacological properties of this ligand-gated receptor and structure-activity relationship of its agonists.

In our present studies, a flexible agonist Carb and a rigid agonist arecolone-MeI were employed. The inward currents generated by both agonists could be detected immediately after the holding potential was applied, lasted for a similar time period and finally diminished. This onset time of the receptor desensitization agrees well with other observations in a similar time period (Harvey and Dryden, 1974 b; Sugiyama *et al.*, 1976; Fiekers *et al.*, 1980; Nojima *et al.*, 1995). Thus, our data suggest that the rigid arecolone-MeI, like the flexible ligands, is capable of inducing receptor desensitization. In term of the schemes proposed by Dunn and Raftery (1997b), therefore, the conformational change to the desensitization state C_1 or C_2 is possible with arecolone-MeI as ligand.

In an earlier study of reconstituted nAchR in giant liposomes (Riquelme *et al.*, 1990), the authors reported currents occurring in 11% of patches in the resting state and this percentage was increased in the presence of Ach, e.g. 50% patches exhibited open-channel activities in the presence of 5 μM Ach. In our study, we found a lower probability of ion-channel open current, in 13.3% cases of Carb and 15.5% cases of arecolone-MeI, respectively. One possible reason is that pH-treated *Torpedo* AchR was used in the earlier

study whereas only crude membrane fragments were used here. Further purification of the receptor by alkali treatment removes peripheral proteins from the receptor complex and this leads to a higher receptor density, i.e. higher ratio of receptors to membrane lipids than that of the crude membranes, and the chances of including a receptor in a patch of membrane are correspondingly less. Alternatively, the receptors may have already desensitized before the recording commenced, or perhaps, even denatured during the liposome making process.

In our investigation, two levels of single-channel open conductance were observed by examining either Carb or arecolone-MeI and they appear to have similar values which is consistent with the concept that channel conductance is independent of the cholinergic agonist used (Tank *et al.*, 1983; Labarca *et al.*, 1984). The main conductances were 56 pS for Carb and 58 pS for areceolone-MeI; the subconductances were 34 pS for Carb and 33 pS for arecolone-MeI. Two conductance classes have been reported by others. For example, by using developing Xenopus muscle cells, Brehm *et al.* (1984) reported two conductances with values of 46 pS and 64 pS; in another study; two conductances were observed to be 35 pS and 54 pS, respectively (Siegelbaum *et al.*,1984). In their examination of reconstituted *Torpedo* receptors, Tank *et al.* (1983) reported two conductances of 42 pS and 12 pS; by investigating mouse BC3H-1 cell, Kullberg *et al.* (1990) found two frequently occurred conductance level to be 50 pS and 12 pS and another occasionally occurred subconductance of 25 pS. Therefore the present data are in good agreement with these previous observations. However, by investigating the properties of *Torpedo* receptors reconstituted in lipid bilayers, some authors suggested that only one conductance could been found (Labarca *et al.*, 1984,1985). It has been suggested that receptor preparation is one of the key factors for the

occurrence of the subconductance state (Steinbach, 1989). Thus their different reconstitution procedure may account for the conductance differences.

It has long been questioned what cause the presence of the subconductance. In a previous study, two independent classes of channels of nAchR were suggested to be the source of conductance and subconductance (Hamill and Sakmann, 1981). Another possibility is that subconductances occur when an agonist molecule binds to a specific site in the lumen which partially occludes the ion channel (Takeda and Trautmann, 1984). It is, however, more likely that the receptor conformational rearrangements of the five subunits, in particular the γ and the δ subunits (Yu *et al.*, 1991), may account for the occurrence of the subconductance (Auerbach and Sachs, 1983; Morris and Montpetit, 1985). The present study provides further evidence to support this notion about conformational changes of the receptor being the principal reason. The main conductance may result from the fully opened channel that have the maximal capacity to allow various cations to permeate through, the subconductance, on the other hand, may be because the conformational changes of the receptor induce a partially opened channel which lowers the ion flux through the channel.

The present results provide evidence to support the kinetic scheme for monoquaternary agonists postulated by Dunn and Raftery (1997b). Even though the pathway of transition from R^*L to R^*L_2 was only observed at the higher ligand concentrations in the fluorescence measurements, it could be detected at lower ligand concentrations when investigated by electrophysiological recordings. 5 μM of Carb, a flexible agonist, can proceed via both pathways, to either R^*L or R^*L_2, in virtually equal frequency which indicates that some channel opening events induced by Carb can reach the

full conductance state by quickly passing the subconductance state R^*L, sometimes the transition between R^*L and R^*L_2 can be observed. On the other hand, most of the channel opening events triggered by arecolone-MeI, a semirigid agonist, remain at the subconductance state R^*L; there are a few cases, however, which approach the main conductance state R^*L_2. These data suggest that both flexible and rigid agonists are able to induce similar conformational changes of the global structure of the receptor molecule and lead to channel opening through the same pathway: the binding of two agonist molecules to the two high affinity sites of the receptor first induces a conformation of the channel resulting in the subconductance state, and the occupancy of the two distinct low affinity (sub)sites by two more molecules may elicit further conformational changes of the receptor to the full conductance state. The rigid structure of arecolone-MeI may prevent the receptor conformation from proceeding to the main conductance state R^*L_2 as the pyridine ring provide some steric hindrance to the receptor, and thus most events recorded are in the lower subconductance state. It was also noted that the desensitization induced by Carb was faster than that by arecolone-MeI which was in good accord with the proposed pathway. Both opening states can lead to receptor desensitization, R^*L proceeds to desensitization state C_1 at a relatively slow rate and R^*L_2 forms C_2 at a faster rate. Since the Carb-induced main and subconductance states occur in virtually equal frequency, receptor desensitization is predicted to be faster for Carb than arecolone-MeI.

It is, however, very difficult to distinguish between two subconductance states of a single channel and the presence of multiple channels opening simultaneously in one patch. Our data show that the higher conductance state was virtually an integral multiple of the

lower class. Thus we cannot rule out the possibility that there were two channels in one patch since *Torpedo* receptors are known to exist as dimers in native membranes (Raftery *et al.*, 1972). However, with carbachol, channel openings were into either the high conductance state (57.1% of the time) or the low conductance state (42.9% of the time) directly from the closed state. It is difficult to accept that two adjacent receptors could be activated exactly in synchrony on the vast majority of occasions that only the high conductance was seen. The occasions where initially low conductance was seen which became high conductance during the duration of the current opening episode and which would be consistent with two adjacent receptors in a patch were in the minority (about 10%). We, therefore, reject as improbable the view that the high conductance state seen in our experiments stemmed from the synchronous opening of two channels in the patch, and hold to the belief that the channel openings observed reflect different conductance levels of a single channel.

Some other rigid agonists, i.e. nicotine, anatoxin and epibatidine, were also found to induce similar single-channel conductance to Ach (Cooper *et al.*, 1996). However, in these studies, only one channel conductance (36 pS) was obtained. The differences of structures and rigidity of these agonists from those of arecolone-MeI and different receptor preparations may account for the differences. More importantly, extremely low concentrations (nanomolar level) of these agonists were used and the value of conductance was similar with the subconductance in the present studies. Thus, it is possible that the conductance in the previous studies is resulted from the binding of the low ligand concentrations to the receptor and subsequently induced conformation of subconductance state R^*L, but not the full

conductance state R^*L_2.

As mentioned in the Introduction, the channel fluctuates between the open state and closed state for a while before the dissociation of the ligand. In our results, the open time of the single channels for both agonists were determined by fitting the open-time histogram with a single exponential. The time constant, τ, of arecolone was larger than that of Carb, indicating that arecolone-induced current had a slightly but not significantly longer duration than that of Carb before it fluctuated to the closed state.

In a recent study, Kawai (1998) reported that the stoichiometry for receptor activation was two for arecolone-MeI, and it binds to the same two high affinity binding site as Ach at equilibrium. Arecolone-MeI was also observed to induce the intrinsic fluorescence changes of the receptor in a concentration-dependent manner with rate constants in keeping with the desensitization process. Therefore the author concluded that arecolone, like other agonists, causes receptor desensitization. The present results of the single-channel recordings agree well with their observations. The reason of why arecolone-MeI does not induce IAS fluorescence changes remains unknown. A earlier report of desensitization study with subunit combinations suggested that the binding site at the α-γ subunit interface may play a more important role in receptor desensitization than that at the α-δ subunit interface (Sine and Claudio, 1991). Even though it does not affect the agonist binding to the receptor and its signal changes upon the binding of agonist reflects the behavior of the receptor as a whole, IAS has been shown to only label half of the binding sites (Dunn *et al.*, 1980). Therefore, Kawai (1998) speculated that the lack of the arecolone-induced IAS fluorescence changes might not be due to the absence of the desensitization process, but due to the

preferential binding of arecolone to the α-δ subunit interface. Alternatively, there may be independent pathways of arecolone-MeI binding and IAS fluorescence changes.

In summary, arecolone-MeI has the same ability as other flexible agonists to cause the receptor desensitization. The onset of receptor desensitization led by arecolone-MeI was at the same time as Carb. When bound with Carb, the receptor undergoes conformational changes to either stay at the subconductance state or quickly pass it to reach the state leading to channel fully opens. On the other hand, when bound with arecolone-MeI, the conformational changes would more likely be stuck at the lower conductance level. Its semirigid structure may account for this difference from that of flexible ligand. Thus, arecolone may be a useful tool for the study of agonist binding structure. Higher concentrations of both ligands and more rigid agonists, e.g. isoarecolone, an analog of arecolone, need to be investigated in the single-channel recording to look for whether they cause receptor desensitization and what conductance they generate.

Reference

Abramson, S.N., Y., Li, P., Culver and P., Taylor. 1989. An analog of lophotoxin reacts covalently with Tyr190 in the α-subunit of the nicotinic acetylcholine receptor. J Biol Chem. 264:12666-12672

Ackermann, and P., Taylor. 1997. Nonidentity of the α-neurotoxin binding sites on the nicotinic acetylcholine receptor revealed by modification in α-neurotoxin and receptor structure. Biochemistry. 36:12836-12844

Akabas, M. H., C., Kaufmann, P., Archdeacon and A., Karlin. 1994. Identification of acetylcholine receptor channel-lining residues in the entire M_2 segment of the α subunit. Neuron. 13:919-927

Anholt, R., J., Lindstrom and M., Montal. 1980. Functional equivalence of monomeric and dimeric forms of purified acetylcholine receptors from *Torpedo californica* in reconstituted lipid vesicles. Eur J Biochem. 109:481-487

Arias, H. R. 1997. Topology of ligand binding sites on the nicotinic acetylcholine receptor Brain Res Rev. 25:133-191

Auerbach A. and F., Sachs. 1983. Flickering of a nicotinic ion channel to a subconductance state. Biophys J. 42:1-10

Aylwin, M. L.and M. M., White. 1994. Gating properties of mutant acetylcholine receptors. Mol Pharmacol. 46:1149-1155

Balass, M., E., Katchalski-Katzir and S., Fuchs. 1997. The α-bungarotoxin binding site on the nicotinic acetylcholine receptor: Analysis using a phage-epitope library. Proc Natl Acad Sci USA. 94:6054-6058

Banerjee, B. and D. K., Ganguly. 1995. Thermodynamic studies with acetylcholine on nicotinic receptors of mammalian skeletal muscle *in vitro*. Biochem Pharmacol. 49:1713-1716

Banerjee, B and D. K., Ganguly. 1996. Thermodynamics of the interaction of *d*-tubocurarine with nicotinic receptors of mammalian skeletal muscle *in vitro*. Eur J Pharmacol. 310:13-17

Barchan, D., M., Ovadia, E., Kochva and S., Fuchs 1995. The binding site of the nicotinic acetylcholine receptor in animal species resistant to α-Bungarotoxin. Biochemistry. 34:9172-9176

Beer, W. H. and E., Reich. 1970. Structure and activity of acetylcholine. Nature. 228:917-922

Behling, R. W., T., Yamane, G., Navon and L. W., Jelinski. 1988. Conformation of acetylcholine bound to the nicotinic acetylcholine receptor. Proc. Natl. Acad. Sci. USA. 85:6721-6725

Betz, H. 1990. Ligand-gated ion channels in the brain: the amino acid receptor superfamily. Neuron. 5:383-392

Blanchard, S. G., S. M. J., Dunn and M. A., Raftery. 1982. Effects of reduction and alkylation on ligand binding and cation transport by *Torpedo californica* acetylcholine receptor. Biochemistry. 21:6258-6264

Blount, P. and J. P., Merlie. 1988. Native folding of an acetylcholine receptor alpha subunit

expressed in the absence of other receptor subunits. J Biol Chem. 263:1072-1080
Blount, P. and J. P., Merlie. 1989. Molecular basis of the two nonequivalent ligand binding sites of the muscle nicotinic acetylcholine receptor. Neuron. 3:349-357
Brehm, P., Y., Kidokoro and F., Moody-Corbett. 1984. Acetylcholine receptor channel properties during development of *Xenopus* muscle cells in culture. J Physiol. 357:203-217
Brisson, A. and P. N. T., Unwin. 1985. Quaternary structure of the acetylcholine receptor. Nature. 315:474-477
Bruns, R. F. 1996. Conformational induction versus conformational selection: evidence from allosteric enhancers. TIPS. 17:189-190
Carter, A. A and R. E., Oswald. 1993. Channel blocking properties of a series of nicotinic cholinergic agonists. Biophys J. 65:840-851
Changeux, J-P., A., Devillers-Thiery and P., Chemouilli. 1984. Acetylcholine receptor: an allosteric protein. Science. 225:1335-1345
Changeux, J-P., J-L., Galzi, A., Devillers-Thiery, D., Bertrand. 1992. The functional architecture of the acetylcholine nicotinic receptor explored by affinity labeling and site-directed mutagenesis. Quar Rev Biophys. 25:395-432
Changeux, J-P. 1995. The acetylcholine receptor: a model for allosteric membrane proteins. Thudichum Medal Lecture. 23:195-205
Chatrenet, B., O., Tremeau, F., Bontems, M. P., Goeldner, C. G., Hirth and A, Ménez. 1990. Topography of toxin-acetylcholine receptor complexes by using photoactivable toxin derivatives. Proc Natl Acad Sci USA. 87:3378-3382
Chen, J., Y-N., Zhang, G., Akk, S. M., Sine and A., Auerbach. 1995. Activation kinetics of recombinant mouse nicotinic acetylcholine receptors: Mutations of α-subunit tyrosine 190 affect both binding and gating. Biophys J. 69:849-859
Cheng, Y. and W., Prusoff. 1973. Relationship between the inhibitory constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. Biochem Pharmacol. 22:3099-3108
Chiara, D. C. and J. B., Cohen. 1997. Identification of amino acids contributing to high and low affinity *d*-tubocurarine sites in the *Torpedo* nicotinic acetylcholine receptor. J Biol Chem. 272:32940-32950
Chinchetru, M. A., J., Marquez, J. C., Garcia-Borron, D. P., Richman and M., Martinez-Carrion. 1989. Interaction of nicotinic acetylcholine receptor with two monoclonal antibodies recognizing different epitopes. Biochemistry. 28:4222-4229
Cohen, J. B., S. D., Sharp and W-S., Liu. 1991. Structure of the agonist binding site of the nicotinic acetylcholine receptor, [^{3}H]acetylcholine mustard identifies residues in the cation-binding subsite. J Biol Chem. 266:23354-23364
Cohen, J. B., M. P., Blanton, D. C., Chiara, S. D. Sharp and B. H., White. 1992. Structural organization of functional domains of the nicotinic acetylcholine receptor. J Cell Biochem. 16E:217-T003
Conti-Tronconi, B. M. and Raftery, M. A. 1982. The nicotinic cholinergic receptor: correlation of molecular structure with functional properties. Annu Rev Biochem. 51:491-530

Conti-Tronconi, B. S., S. M. J., Dunn and M., A., Raftery. 1982. Independent sites of low and high affinity for agonists on *Torpedo californica* acetylcholine receptor. Biochem Biophys Res Commu.107:123-129

Conti-Tronconi, B. M. and M. A., Raftery. 1986. Nicotinic acetylcholine receptor contains multiple binding sites: evidence from binding of α-dendrotoxin. Proc Natl Acad Sci USA. 83:6646-6650

Conti-Tronconi, B. M., F., Tang, S., Walgrave, W., Gallagher. 1990a. Nonequivalence of alpha-bungarotoxin binding sites in the native nicotinic receptor molecule. Biochemistry. 29:1046-4054

Conti-Tronconi, B. M., F., Tang, B. M., Diethelm, S. R., Spencer, S., Reinhardt-Maelicke, A., Maelicke. 1990b. Mapping of a cholinergic binding site by means of synthetic peptides, monoclonal antibodies, and alpha-bungarotoxin. Biochemistry. 29:6221-6230

Conti-Tronconi, B. M., K. E., McLane, M. A., Raftery, S. A., Grando and M. P., Protti. 1994. The nicotinic acetylcholine receptor: structure and autoimmune pathology. Crit Rev Biochem Mol Biol. 29:69-123

Cooper, J. C., Gutbrod, O., Wizemann, V. and Methfessel, C. 1996. Pharmacology of the nicotinic acetylcholine receptor from fetal rat muscle expressed in *Xenopus* oocytes. Eur J Pharmacol. 309:287-298

Criado, M and B. U., Keller. 1987. A membrane fusion strategy for single-channel recordings of membranes usually non-accessible to patch-clamp pipette electrodes. FEBS. 224:172-176

Culver, P., W., Fenical and P., Taylor. 1984. Lophotoxin irreversibly inactivates the nicotinic acetylcholine receptor by preferential association at one of the two primary agonist sites. J Biol Chem. 259:3763-3770

Czajkowski, C. and A., Karlin. 1991. Agonist binding site of *Torpedo* electric tissue nicotinic acetylcholine receptor: a negatively charged region of the δ subunit within 0.9 nm of the α subunit binding site disulfide. J Biol Chem. 266:22603-22612

Czajkowski, C., C., Kaufmann and A., Karlin. 1993. Negatively charged amino acid residues in the nicotinic receptor δ subunit that contribute to the binding of acetylcholine. Proc Natl Acad Sci USA. 90:6285-6289

Czajkowski, C. and A., Karlin. 1995. Structure of the nicotinic receptor acetylcholine-binding site. J Biol Chem. 270:3160-3164

Deneris, E. S., J., Boulter, J., Connolly, E., Wada, K., Wada, D., Goldmann, L. W., Swanson, J., Patrick and S., Heinemann. 1989. Genes encoding neuronal nicotinic acetylcholine receptors. Clin Chem. 35:731-737

Dennis, M., J., Giraudat, F., Kotzyba-Hibert, M., Goeldner, C., Hirth, J-Y., Chang and J-P., Changeux. 1986. A photoaffinity ligand of the acetylcholine-binding site predominantly labels the region 179-207 of the α-subunit on native acetylcholine receptor from *Torpedo marmorata*. FEBS Lett. 207:243-249

Dennis, M., J., Giraudat, F., Kotzyba-Hibert, M., Goeldner, C., Hirth, J-Y., Chang, C., Lazure, M., Chretien and J-P., Changeux. 1988. Amino acids of the *Torpedo marmorata* acetylcholine receptor subunit labeled by a photoaffinity ligand for the

acetylcholine binding site. Biochemistry 27:2346-2357
Devillers-Thiery A, J-L., Galzi, J. L., Eisele, S., Bertrand, D., Bertrand and J-P., Changeux. 1993. Functional architecture of the nicotinic acetylcholine receptor: a prototype of ligand-gated ion channels; J Membrane Biol. 136: 97-112
Dougherty D. A and D. A., Stauffer. 1990. Acetylcholine binding by a synthetic receptor: Implication for biological recognition. Science. 250:1558-1560
Dougherty D. A. 1996. Cation-π interactions in chemistry and biology: A new view of benzene, Phe, Tyr, and Trp. Science. 271:163-168
Dunn, S. M. J., S. G., Blanchard and M. A., Raftery. 1980. Kinetics of Carbamylcholine binding to membrane-bound acetylcholine receptor monitored by fluorescence changes of a covalently bound probe. Biochemistry. 19:5645-5652
Dunn, S. M. J., S. G., Blanchard and M. A., Raftery. 1981. Effects of local anesthetics and histrionicotoxin on the binding of carbamoylcholine to membrane-bound acetylcholine receptor. Biochemistry. 20:5617-5624
Dunn, S. M. J. and M. A., Raftery. 1982a. Activation and desensitization of *Torpedo* acetylcholine receptor: evidence for separate binding sites. Proc Natl Acad Sci USA. 79:6757-6761
Dunn, S. M. J. and M. A., Raftery. 1982b. Multiple binding sites for agonists on *Torpedo californica* acetylcholine receptor. Biochemistry. 21:6264-6271
Dunn, S. M. J., B. M., Conti-Tronconi and M. A., Raftery. 1983. Separate sites of low affinity for agonists on *Torpedo californica* acetylcholine receptor. Biochemistry. 22:2512-2518
Dunn, S. M. J., B.M., Conti-Tronconi and M.A., Raftery. 1986. Acetylcholine receptor dimers are stabilized by extracellular disulfide bonding. Biochem Biophys Rev Commu. 139:830-837
Dunn, S. M. J., 1993. Structure and function of the nicotinic acetylcholine receptor. Advance in Structural Biology. 2: 225-244
Dunn, S. M. J., B.M., Conti-Tronconi and M.A., Raftery.1993. A high-affinity site for acetylcholine occurs to the α-γ subunit interface of *Torpedo* nicotinic acetylcholine receptor. Biochemistry. 32: 8616-8621
Dunn, S. M. J. and M. A., Raftery. 1993. Cholinergic binding sites on the pentameric acetylcholine receptor of *Torpedo californica*. Biochemistry. 32:8608-8615
Dunn, S. M. J., A. N., Bateson and I. L., Martin. 1994. Molecular neurobiology of the $GABA_A$ receptor. Int Rev Neurobiol. 36:51-96
Dunn, S. M. J. and M. A., Raftery. 1997a. Agonist binding to the *Torpedo* acetylcholine receptor. 1. Complexities revealed by dissociation kinetics. Biochemistry. 36:3846-3853
Dunn, S. M. J. and M. A., Raftery. 1997b. Agonist binding to the *Torpedo* acetylcholine receptor. 2. Complexities revealed by association kinetics. Biochemistry. 36:3854-3863
Dwyer, T. M., D. A., Adams and B., Hille. 1980. The permeability of the endplate channel to organic cations in frog muscle. J Gen Physiol. 75:469-492
Elliott, J., S.G., W., Blanchard, J., Wu, C.D., Miller, P., Strader, H-P., Hartig, J., Moore,

M.A., Racs, M.A., Raftery. 1980. Purification of *Torpedo californica* post-synaptic membranes and fractionation of their constituent proteins. Biochem J. 185:667-677

Fels, G., E. K. Wolff and A., Maelicke. 1982. Equilibrium binding of acetylcholine to the membrane-bound acetylcholine receptor. Eur. J Biochem. 127:31-38

Fiekers, J. F., P. M., Spannbauer, B., Scubon-Mulieri and R. I., Parsons. 1980. Voltage dependence of desensitization: influence of calcium and activation kinetics. J Gen Physiol. 75:511-529

Fieser, L. F. and M., Fieser. 1957. Introduction to organic chemistry. Harvard University Press

Filatov, G. N. and M. M., White. 1995. The role of conserved leucine in the M_2 domain of acetylcholine receptor in channel gating. Mol Pharmacol. 48:379-384

Fraenkel, Y., M., Gershoni and G., Navon. 1994. NMR analysis reveals a positively charged hydrophobic domain as a common motif to bound acetylcholine and d-tubocurarine. Biochemistry. 33:644-650

Fu, D-X and S. M., Sine. 1994. Competitive antagonists bridge the α-γ subunit interface of the acetylcholine receptor through quaternary ammonium-aromatic interactions. J Biol Chem. 269:26152-26157

Fucile, S., A.M., Mileo, F., Grassi, A. M., Salvatore, S., Alemá and F., Eusebi. 1996. Identification of a determinant of AchR gating kinetics in the extracellular portion of the γ subunit. Eur J Neurosci. 8:2564-2570

Galzi J-L, F., Revah, D., Black, M., Goeldner, C., Hirth and J-P., Changeux. 1990. Identification of a novel amino acid α-tyrosine 93 within the cholinergic ligands-binding sites of the acetylcholine receptor by photoaffinity labeling. Additional evidence for a three-loop model of the cholinergic ligands-binding sites. J Biol Chem. 265:10430-10437

Galzi, J-L., F., Revah, A., Bessis and J-P., Changeux. 1991a. Functional architecture of the nicotinic acetylcholine receptor: From electron organ to brain. Annu Rev Pharmacol Toxicol. 31: 37-72

Galzi, J-L, F., Revah, F., Bouet, A., Menez, M., Goeldner, C., Hirth and J-P., Changeux. 1991b. Allosteric transitions of the acetylcholine receptor probed at the amino acid level with a photolabile cholinergic ligand; Proc Natl Acad Sci USA. 88: 5051-5055

Galzi, J-L., D., Bertrand, A., Devillers-Thiery, F., Revah, S., Bertrand and J-P., Changeux. 1991c. Functional significance of aromatic amino acids from three peptide loops of the α-7 neuronal nicotinic receptor site investigated by site directed mutagenesis. FEBS Lett. 294:198-202

Giraudat, J., M., Dennis, T., Heidmann, J. Y., Chang and J-P., Changeux. 1986. Structure of the high affinity binding site for noncompetitive blockers of the acetylcholine receptor: Serine-262 of the δ subunit is labeled by [^{3}H]chlorpromazine. Proc Natl Acad Sci USA. 83:2719-2723

Grassi, F., O., Epifano, A. M., Mileo, B., Barabino and F., Eusebi. 1998. The open duration of fetal Ach receptor-channel changes during mouse muscle development. J Physiol. 508:393-400

Grenningloh, G., A., Rienitz, B., Schmitt, C., Methfessel, M., Zensen, K., Beyrenther, E. D.,

Gundelfinger and H., Betz. 1987. The strychnine-binding subunit of glycine receptor shows homology with nicotinic acetylcholine receptor. Nature. 328:215-220

Gu, Y., J. R., Forsayeth, S., Verrall, X-M., Yu and Z. W., Hall. 1991. Assembly of the mammalian muscle acetylcholine receptor in transfected COS cells. J Cell Biol. 114:799-807

Hamill, O. P. and B., Sakmann. 1981. Multiple conductance states of single acetylcholine receptor channels in embryonic muscle cells. Nature. 294:462-464

Hamill, O. P., A., Marty, E., Neher, B., Sakmann and F. J., Sigworth. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflügers Archiv. 391:85-100

Harvey, A. L. and W. F., Dryden. 1974a. The action of some anticholinesterase drugs on skeletal muscle in culture. J Pharmacy Pharmacol. 26:865-870

Harvey, A. L. and W. F., Dryden. 1974b. Depolarization, desensitization and the effects of tubocurarine and neostigmine in cultured skeletal muscle. Eur J Pharmacol. 27:5-13

Herlitze, S., A., Villarroel, V., Witzemann, M., Koenen and B., Sakmann. 1996. Structural determinants of channel conductance in fetal and adult rat muscle acetylcholine receptor. J Physiol. 492:775-787

Hille, B. 1992. Ionic channels of excitatory membranes. 2nd edition, Sinauer Associations, Sunderland MA

Hucho, F., G., Bandini and B. A., Suarez-Isla. 1978. The acetylcholine receptor as part of a protein complex in receptor-enriched membrane fragments from *Torpedo californica* electric tissue. Eur J Biochem. 83:335-340

Hucho, F., Oberthür and F., Lottspeich. 1986. The ion channel of the nicotinic acetylcholine receptor is formed by the homologous helices MII of the receptor subunits. FEBS Lett. 205:137-142

Hucho, F., V.I., Tsetlin, and J., Machold. 1996. The emerging three-dimensional structure of a receptor: the nicotinic acetylcholine receptor. Eur J Biochem. 239: 539-557

Huganir, R.L., M., Schell and E., Racker. 1979. Reconstitution of the purified acetylcholine receptor from *Torpedo californica*. FEBS Lett. 108: 155-160

Huganir, R. L. and P., Greengard. 1987. Regulation of receptor function by protein phosphorylation. TIPS. 8:472-477

Imoto, K, C., Methfessel, B., Sakmann, M., Mishina, Y. Mori, T., Konno, K., Fukuda, M., Kurasaki, H., Bujo, Y., Fujita and S., Numa. 1986. Location of a delta-subunit region determining ion transport through the acetylcholine receptor channel. Nature. 324:670-674

Imoto, K., C., Busch, B., Sakmann, M., Mishina, T., Konno, J., Nakai, H., Bujo, Y., Mori, K., Fukuda and S., Numa. 1988. Rings of negatively charged amino acids determine the acetylcholine receptor channel conductance. Nature. 335:645-648

Kachalski, S. G., B. S., Jensen, D., Barchan and S., Fuchs. 1995. Two subsites in the binding domain of the acetylcholine receptor: An aromatic subsite and a proline subsite. Proc Natl Acad Sci USA. 92:10801-10805

Kao, P. N., A. J., Dwork, R. R., Kaldany, M. L., Silver, J., Wideman, S., Stein and A., Karlin. 1984. Identification of the α-subunit half cysteine specifically labeled by an

affinity reagent for the acetylcholine receptor binding site. J Biol Chem. 259:11662-11665

Kao, P. N. and A., Karlin. 1986. Acetylcholine receptor binding site contains a disulfide cross-link between adjacent half-cystinyl residues. J Biol Chem. 261:8085-8088

Karlin, A., E., Holtzman, N., Yodh, P. Lobel, J., Wall and J., Hainfeld. 1983. The arrangement of the subunits of the acetylcholine receptor of *Torpedo californica*. J Biol Chem. 258:6678-6681

Karlin, A., 1993. Structure of nicotinic acetylcholine receptor; Curr Opin Neurobiol. 3: 299-309

Karlin, A. and M. H., Akabas. 1995. Toward a structural basis for the function of nicotinic acetylcholine receptor and their cousins; Neuron. 15: 1231-1244

Katz, B. and S., Thesleff. 1957. A study of the "Desensitization" produced by acetylcholine at the motor end-plate. J Physiol. 138:63-80

Katz, B. and Miledi, R. 1971. Further observations on acetylcholine noise. Nature. 232:124-126

Katz, B. and Miledi, R. 1977. Transmitter leakage from motor nerve endings. Proc R Soc London Ser B 196:59-72

Kawai, H. 1998. PhD thesis. University of Minnesota, St Paul, MN

Kearney, P.C., H. Y., Zhang, W. G., Zhong, D. A., Dougherty and H.A., Lester. 1996. Determinants of nicotinic receptor gating in natural and unnatural side chain structure at the MII 9' position. Neuron. 17:1221-1229

Keller, B. U., R., Hedrich, W. L., Vaz and M., Criado. 1988. Single channel recordings of reconstituted ion channel proteins: an improved technique. Pflügers Arch. 411:94-100

Kenakin, T. 1995. Agonist-receptor efficacy I: mechanism of efficacy and receptor promiscuity. TIPS. 16:188-192

Kenakin, T. 1996 Receptor conformational induction versus selection: all part of the same energy landscape. TIPS. 17:190-191

Kistler, J., R. M., Stroud, M. W., Klymkowsky, R. A., Lalancette and R. H., Fairclough. 1982. Structure and function of an acetylcholine receptor. Biophys J. 37:371-383

Klarsfeld, A., A., Devillers-Thiery, J., Giraudat, and J-P., Changeux,. 1984. A single gene codes for the nicotinic acetylcholine receptor alpha-subunit in *Torpedo marmorata*: structure and developmental implications. EMBO J. 3:35-41

Kreienkamp, H-J., R. K., Maeda, S. M., Sine and P., Taylor. 1995. Intersubunit contacts governing assembly of the mammalian nicotinic acetylcholine receptor. Neuron. 14:635-644

Kubalek, E., S., Ralston, J., Lindstrom and N., Unwin. 1987. Location of subunits within the acetylcholine receptor by electron image analysis of tubular crystals from *Torpedo marmorata*. J Cell Biol. 105:9-18

Kullberg, R., Owens, J. L., Comacho, P., Mandel, G. and Brehm, P. 1990. Multiple conductance classes of mouse nicotinic acetylcholine receptors expressed in *Xenopus* oocytes. Proc Natl Acad Sci USA. 87:2067-2071

Labarca, P., J., Lindstrom and M., Montal.1984. The acetylcholine receptor channel from *Torpedo californica* has two open states. J Neurosci. 4:502-507

Labarca, P., M. S., Montal, J. M., Lindstrom and M.,Montal. 1985. The occurrence of long openings in the purified cholinergic receptor channel increase with acetylcholine concentration. J Neurosci. 5:3409-3413

Labarca, C., M. W., Nowak, H-Y., Zhang, L-X., Tang, P., Deshpande and H. A., Lester. 1995. Channel gating governed symmetrically by conserved leucine residues in the M_2 domain of nicotinic acetylcholine receptor. Nature. 376:514-516

Lena C and Changeux J-P. 1993. Allosteric modulations of the nicotinic acetylcholine receptor; TINS. 16: 181-186

Lentz , T. L. 1995. Differential binding of nicotine and α-bungarotoxin to residues 173-204 of the nicotinic acetylcholine receptor α1 subunit. Biochemistry. 34:1316-1322

Leonard, R. J., C. G., Labarca, P., Charnet, N., Davidson and H. A., Lester. 1988. Evidence that the M_2 membrane-spanning region lines the ion channel pore of the nicotinic receptor. Science. 242:1578-1581

Machold, D., Y., Utkin, D., Kirsch, R., Kaufmann, V., Tsetlin and F., Hucho. 1995. Photolabeling reveals the proximity of α-Neurotoxin binding site to the M_2-helix of the ion channel in the nicotinic acetylcholine receptor. Proc Natl Acad Sci USA. 92:7282-7286

Magleby, K. L. and C. F., Stevens. 1972. The effect of voltage on the time course of end-plate currents. J Physiol. 223:151-171

Maricq, A. V., Peterson, A., Brake, A., Myers, R. and Julius, D. 1991. Primary structure and functional expression of the $5HT_3$ receptor, a serotonin-gated ion channel. Science. 254:432-437

Martin, M. D. and A., Karlin. 1997. Functional effects on the acetylcholine receptor of multiple mutations of γAsp174 and δAsp180. Biochemistry. 36:10742-10750

McCarthy, M. P., J. P., Earnest, E. F. Young, S., Choe and R. M., Stroud. 1986. The molecular neurobiology of the acetylcholine receptor. Annu Rev Neurosci. 9:383-413

McLane, K. E., F., Tang and B. M., Conti-Tronconi. 1990. Location of sequence segments forming a kappa-bungarotoxin-binding site on the α3 neuronal acetylcholine receptor. J Biol Chem. 265:1537-1544

McLane, K. E., X., Wu and B. M., Conti-Tronconi. 1991a. Structural determinants of α-bungarotoxin binding to the sequence segment 181-200 of the muscle nicotinic acetylcholine receptor α-subunit: effects of cysteine/cysteine modification and species-specific amino acid substitutions. Biochemistry. 30:4925-4934

McLane, K. E., R., Schoepfer, X., Wu, J. M., Lindstrom and B. M., Conti-Tronconi. 1991b. Identification of sequence segments forming the α-bungarotoxin binding sites on two nicotinic acetylcholine receptor α-subunits from the avian brain. J Biol Chem. 266:15230-15239

McLane, K. E., X-D., Wu and B. M., Conti-Tronconi.1994. An α-bungarotoxin-binding sequence on the *Torpedo* nicotinic acetylcholine receptor α-subunit: Conservative amino acid substitutions reveal side-chain specific interactions. Biochemistry. 33:2576-2585

McLane, K. E., Dunn, S. M. J., Manfred, A. A., Conti-Tronconi, B.M. and Raftery, M. A. 1996. The nicotinic acetylcholine receptor as a model for a superfamily of ligand-gated ion channel proteins. in Protein Engineering Design (ed. Carey, P. R.)

Academic press
Merlie, J. P., R., Sebbane, S., Gardner and J., Lindstrom. 1983. cDNA clone for the alpha-subunit of the acetylcholine receptor from the mouse muscle cell line BC_3H-$_1$. Proc Natl Acad Sci USA. 80:3845-3849
Middleton, R.E. and J. B., Cohen. 1991. Mapping of the acetylcholine binding site of the nicotinic acetylcholine receptor: [^{3}H]nicotine as an agonist photoaffinity label. Biochemistry. 30:6987-6997
Mihovilovic, M. and D. P., Richman. 1987. Monoclonal antibodies as probes of the α-bungarotoxin and cholinergic binding regions of the acetylcholine receptor. J Biol Chem. 262:4978-4986
Mileo, A. M., L., Monaco, E., Palma, F., Grassi, R., Miledi and F., Eusebi. 1995. Two forms of acetylcholine receptor γ subunit in mouse muscle. Proc Natl Acad Sci USA. 92:2686-2690
Miller, C. 1989. Genetic manipulation of ion channel: A new approach to structure and mechanism. Neuron. 2:1195-1205
Mishina, M., T., Tobimatsu, K., Imoto, K., Tanaka, Y., Fujita, K., Fukuda, M., Murasaki, T., Takahashi, M., Kuno and S., Numa. 1985. Location of functional region of acetylcholine receptor α-subunit by site-directed mutagenesis. Nature. 313:364-369
Mishina, M., T., Takai, K., Imoto, M., Noda, T., Takahashi, S., Numa, C., Methfessel and B., Sakmann. 1986. Molecular distinction between fetal and adult forms of muscle acetylcholine receptor. Nature. 321:406-411
Mitra, A. K., M. P., McCarthy and R. M., Stroud. 1989: Three-dimensional structure of the nicotinic acetylcholine receptor and location of the major associated 43 KD cytoskeletal protein, determined at 22 Å by low dose electron microscopy and x-ray diffraction to 12.5 Å. J Cell Biol. 109:755-774
Moore, H-P. H., P. R., Hartig and M. A. Raftery. 1979. Correlation of polypeptide composition with functional events in acetylcholine receptor-enriched membranes from *Torpedo californica*. Proc Natl Acad Sci USA. 76:6265-6269
Moore, H-P. H. and M. A., Raftery. 1979. Ligand-induced interconversion of affinity states in membrane-bound acetylcholine receptor from *Torpedo californica*. Effects of sulfhydryl and disulfide reagents. Biochemistry. 18:1907-1911
Morris, C. and M., Montpetit. 1985. Multiple conductance states of the acetylcholine receptor channel complex. Can J Physiol Pharmacol. 64:347-355
Neher, E. and B., Sakmann. 1976. Single-channel currents recorded from membrane of denervated frog muscle fibres. Nature. 260:799-801
Neher, E. and B., Sakmann. 1992. The patch clamp technique. Science American March
Neubig, R. R., E. K., Krodel, N. D., Boyd and J. B., Cohen. 1979. Acetylcholine and local anaesthetics binding to *Torpedo* nicotinic postsynaptic membranes after removal of nonreceptor peptides. Proc Natl Acad Sci USA. 76:690-694
Neubig, R. R. and J. B., Cohen. 1979. Equilibrium binding of [^{3}H]tubocurarine and [^{3}H]acetylcholine by *Torpedo* postsynaptic membranes: stoichiometry and ligand interaction. Biochemistry. 18: 5464-5475
Neubig, R.R., N. D., Boyd and J. B., Cohen. 1982. Conformations of *Torpedo* acetylcholine receptor associated with ion transport and desensitization. Biochemistry. 21:3460-

3467
Noda, M., H., Takahashi, T., Tanabe, M., Toyosato, Y., Furutani, T., Hirose, M., Asai, S., Inayama, T., Miyata and S., Numa. 1982. Primary structure of α-subunits precursor of *Torpedo californica* acetylcholine receptor deduced from cDNA sequence. Nature. 299:793-797
Noda, M., H., Takahashi, T., Tanabe, M., Toyosato, S., Kikyotani, T., Hirose, M., Asai, H., Takashima, S., Inayama, T., Miyata and S., Numa. 1983a. Primary structure of β- and δ-subunit precursor of *Torpedo californica* acetylcholine receptor deduced from cDNA sequence. Nature. 301:251-255
Noda, M., H., Takahashi, T., Tanabe, M., Toyosato, Y., Furutani, T., Hirose, H., Takashima, M., Asai, S., Inayama, T., Miyata and S., Numa. 1983b. Structural homology of *Torpedo californica* acetylcholine receptor subunits. Nature. 302:528-532
Nojima, H., H., Tseuneki, I., Kimura and M., Kimura. 1995. Accelerated desensitization of nicotinic receptor channels and its dependence on extracellular calcium in isolated skeletal muscles of streptozotocin-diabetic mice. Br J Pharmacol. 1680-1684
Ochoa, E. L., A.,Chattopadhyay, and M. G., McNamee. 1989. Desensitization of the nicotinic acetylcholine receptor: molecular mechanisms and effect of modulators. Cellular Mol Neurobiol. 9: 141-178
Ogden, D. C. and D., Colquhoun. 1985. Ion channel block by acetylcholine, carbachol and suberyldicholine at the frog neuromuscular junction. Proc R Soc Lond. B225:329-355
Okonjo, K. O., J., Kuhlmann and A., Maelicke. 1991. A second pathway of activation of the *Torpedo* acetylcholine receptor. Eur J Biochem. 200:671-677
O'Leary, M. E. and M. M., White. 1992. Mutation analysis of ligand-induced activation of the *Torpedo* acetylcholine receptor. J Biol Chem. 267:8360-8365
O'Leary, M. E., G. N., Filatov and M. M., White. 1994. Characterization of d-tubocurarine binding of *Torpedo* acetylcholine site. Am J Physiol. 266:C648-653
Paradiso, K. and P., Brehm. 1998. Long-term desensitization of nicotinic acetylcholine receptors is regulated via protein canasta A-mediated phosphorylation. J Neurosci. 15:9227-9237
Pedersen, S. E. and J. B., Cohen. 1990. d-Tubocurarine binding sites are located at α-γ and α-δ subunit interfaces of the nicotinic acetylcholine receptor. Proc Natl Acad Sci USA. 87:2785-2789
Popot J. L., J., Cartaud and J-P., Changeux. 1981. Reconstitution of a functional acetylcholine receptor. Incorporation into artificial lipid vesicles and pharmacology of the agonist-controlled permeability changes. Eur J Biochem 118: 203-214
Quast, U., M. I., Schimerlik and M. A., Raftery. 1979. Ligand-induced changes in membrane-bound acetylcholine receptor observed by ethidium fluorescence. 2. Stopped-flow studies with agonists and antagonists. Biochemistry. 18:1891-1901
Raftery, M.A., J., Schmidt and D. G., Clark. 1972. Specificity of α-Bungarotoxin binding to *Torpedo californica* electroplax. Arch Biol Chem Biophys. 152:882-886
Raftery, M. A., M., Hunkapillar, C. D., Strader, and L. E., Hood. 1980. Acetylcholine receptor: complex of homologous subunits. Science. 208:1454-1456
Raftery, M. A., S. M. J., Dunn, B. W., Conti-Tronconi, D., Middlemas and R. D., Crawford

1983. Cold Spring Harbor Symp Quant Biol. 48:21-33

Raftery, M. A., B. W., Conti-Tronconi, S. M. J., Dunn, D., Middlemas and R. D., Crawford. 1984. The nicotinic acetylcholine receptor: Its structure, multiple binding sites, and cation transport properties. Functional & Applied Toxicol. 4:S34-51

Raftery, M.A., B. M., Conti-Tronconi and S. M. J., Dunn. 1985. Structural and functional aspects of the nicotinic acetylcholine receptor. Functional & Applied Toxicol. 5:S39-46

Revah, F., J-L., Galzi, J., Giraudat, P.Y., Haumont, F., Lederer and J-P., Changeux. 1990. The noncompetitive blocker [^{3}H]chlorpromazine labels three amino acids of the acetylcholine receptor γ subunit: implication for the α helical organization of the MII segments and the structure of the ion channel. Proc Natl Acad Sci USA. 87:4675-4679

Riquelme, G., E., Lopez, L. M., Garcia-Segura, J. A., Ferragut and J. M., Gonzalez-Ros. 1990. Giant liposomes: a model system in which to obtain patch-clamp recordings of ionic channels. Biochemistry. 29:11215-11222

Sakmann, B., C., Methfessel, M., Mishina, T., Takahashi, T., Takai, M., Kurasaki, K., Fukuda and S., Numa. 1985. Role of acetylcholine receptor subunits in gating of the channel. Nature. 318:538-543

Sakmann, B. 1992. Elementary steps in synaptic transmission revealed by currents through single ion channels. Science. 256:503-512

Sankararamakrishnan, R., C., Adcock and S. P., Sansom. 1996. The pore domain of the nicotinic acetylcholine receptor: Molecular modeling, pore dimensions, and electrostatics. Biophys J. 71:1659-1671

Sargent, P. B. 1993. The diversity of neuronal nicotinic acetylcholine receptors. Annu Rev Neurosci. 16:403-443

Schofield, P. R., M. G., Darlison, N., Fujita, D. R., Burt, P.A., Stephenson, H., Rodriguez, L. M., Rhee, J., Ramachandran, V., Reale, T. A., Glencorse, P.H., Seeburg and E. A., Barnard. 1987. Sequence and functional expression of the $GABA_A$ receptor shows a ligand-gated receptor superfamily. Nature 328:221-227

Siegelbaum, S., A., Trautmann and J., Koenig. 1984. Single acetylcholine-activated channel currents in developing muscle cells. Dev. Biol. 104:366-379

Sine, S. M. and J. H., Steinbach. 1984. Agonists block currents through acetylcholine receptor channels. Biophys J. 46:277-284

Sine, S. M., T., Claudio and F. J., Sigworth. 1990. Activation of *Torpedo* acetylcholine receptors expressed in mouse fibroblasts. Single channel current kinetics reveal distinct agonist binding affinities. J Gen Physiol. 96:395-437

Sine, S. M. and T., Claudio. 1991. γ- and δ-subunits regulate the affinity and the cooperativity of ligand binding to the acetylcholine receptor. J Biol Chem. 266:19369-19377

Sine, S. M. 1993. Molecular dissection of subunit interfaces in the acetylcholine receptor: identification of residues that determine curare selectivity. Proc Natl Acad Sci USA. 90: 9436-9440

Sine, S. M., P., Quiram, F., Papanicolan, H-J., Kreienkamp and P., Taylor. 1994. Conserved tyrosines in the α-subunit of the nicotinic acetylcholine receptor stabilize quaternary

ammonium groups of agonists and curariform antagonists. J Biol Chem. 269:8808-8816

Spivak, C. E., J. A., Waters, B., Witkop and E. X., Albuquerque. 1983. Potencies and channel properties induced by semirigid agonists at frog nicotinic acetylcholine receptors. Mol Pharmacol. 23:337-343

Spivak, C. E., J. A., Waters and R. B., Aronstam. 1989. Binding of semirigid nicotinic agonists to nicotinic and muscarinic receptors. Mol Pharmacol. 36:177-184

Stauffer, D. A. and A., Karlin. 1994. Electrostatic potential of the acetylcholine binding sites in the nicotinic receptor probed by reactions of binding-site cysteines with charged methanethiosulfonates. Biochemistry. 33:6840-6849

Steinbach, J. H. 1989. Structural and functional diversity in vertebrate skeletal muscle nicotinic acetylcholine receptors. Annu Rev Physiol. 51:353-365

Stroud, R.M., M.P., McCarthy and M., Shuster. 1990. Nicotinic acetylcholine receptor superfamily of ligand-gated ion channels. Biochemistry. 29: 11009-11023

Suarez-Isla, B. A. and F., Hucho. 1977. Acetylcholine receptor: SH group reactivity as indicator of conformational changes and functional states. FEBS Lett. 76:65-69

Sugiyama, H., J-L., Popot and J-P., Changeux. 1976. Studies on the electrogenic action of acetylcholine with *Torpedo marmorata* electric organ. II. Pharmacological desensitization *in vitro* of the receptor-rich membrane fragments by cholinergic agonists. J Mol Biol. 106:485-496

Sugiyama, N., A. E., Boyd and P., Taylor. 1996. Anionic residue in the α-subunit of the nicotinic acetylcholine receptor contributing to subunit assembly and ligand binding. J Biol Chem. 271:26575-26581

Sussman, J. L., M., Harel, F., Frolow, C., Oefner, A., Goldman, L., Toker and I., Silman. 1991. Atomic structure of acetylcholinesterase from *Torpedo californica*: a prototypic acetylcholine binding protein. Science. 253:872-879

Takai, T., M., Noda, M., Mishina, S., Shimizu, Y., Furutani, T., Kayano, T., Ikeda, T., Kubo, H., Takahashi, T., Takahashi, M., Kuno and S., Numa. 1985. Cloning, sequencing and expression of cDNA for a novel subunit of acetylcholine receptor from calf muscle. Nature. 315:761-764

Takeda, K and A., Trautmann. 1984. A patch-clamp study of the partial agonist actions of tubocurarine on rat myotubes. J Physiol. 349:353-374

Tank, D. W., R. L., Huganir, P., Greengard and W. W., Webb. 1983. Patch-recorded single-channel currents of the purified and reconstituted *Torpedo* acetylcholine receptor. Proc Natl Acad Sci USA. 80:5129-5133

Tine, S-J. and M. A., Raftery. 1993. Photoaffinity labeling of *Torpedo* acetylcholine receptor at multiple sites. Proc Natl Acad Sci USA. 90:7308-7311

Tomaselli, G. F., J. T., McLaughlin, M. E., Jurman, E., Howrot and G., Yellen. 1991. Mutations affecting agonist sensitivity of the nicotinic acetylcholine receptor. Biophys J. 60:721-727

Tsigelny, I., N., Sugiyama, S. M., Sine and P., Taylor. 1997. A model of the nicotinic receptor extracellular domain based on sequence identity and residue location. Biophys J. 73:52-66

Unwin, N. 1993. Nicotinic acetylcholine receptor at 9 Å resolution. J Mol Biol. 229: 1101-

1124
Unwin, N. 1995. Acetylcholine receptor channel imaged in the open state. Nature 373:37-43
Unwin, N. 1996. Projection structure of the nicotinic acetylcholine receptor: distinct conformations of the α subunits. J Mol Biol. 257:586-596
Valenzuela C.F., P., Weigh, J., Yguerabide, D.A., Johnson. 1994. Transverse distance between the membrane and the agonist binding sites on the *Torpedo* acetylcholine receptor: A fluorescence study. Biophys J. 66:674-682
Watters, D. and A., Maelicke. 1983. Organization of ligand binding sites at the acetylcholine receptor: a study with monoclonal antibodies. Biochemistry 22:1811-1819
White, B. M. and J. B.Cohen. 1992. Agonist-induced changes in the structure of the acetylcholine receptor M_2 region revealed by photoincorporation of an uncharged nicotinic noncompetitive antagonist. J Biol Chem. 267:15770-15783
Wu, G., and K.W., Miller. 1994. Ethanol enhances agonist-induced fast desensitization in nicotinic acetylcholine receptor. Biochemistry. 33:9085-9091
Wu, W. C. and M.A., Raftery. 1981. Reconstitution of acetylcholine receptor function using purified receptor protein. Biochemistry 20: 694-701
Yu, L., J., La Polla and N., Davidson. 1986. Mouse muscle acetylcholine receptor γ subunit: cDNA sequence and gene expression. Nucleic Acids Res. 14:3539-3555
Yu, L., R. J., Leonard, N., Davidson and H. A., Lester. 1991. Single-channel properties of mouse-*Torpedo* acetylcholine receptor hybrids expressed in *Xenopus* oocytes. Mol Brain Res. 10:203-211
Zhang, Y., J., Chen and A., Auerbach. 1995. Activation of recombinant mouse acetylcholine receptors by acetylcholine, carbamylcholine and tetramethylammonium. J Physiol. 486:189-206