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Highly Mobile Channel Lining Transmembrane Segments in Muscle Nicotinic Acetylcholine Receptors?

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The Cys-Loop gene super-family of ligand gated ion channels includes nicotinic acetylcholine (nACh), gamma aminobutyric acid ($GABA_A$), glycine (Gly), and serotonin ($5\text{-}HT_{3A}$) receptors. Each receptor is either a homo- or heteropentamer made up of 5 identical or homologous subunits. Each subunit has an extracellular N-terminal domain, which houses the ligand binding site; a transmembrane domain which spans the membrane four times as α-helical segments (M1-M4); and a long loop between M3 and M4 that constitutes the intracellular domain. M2 lines the ion channel, whereas M1, M3 and M4 are abluminal.

Muscle nAChRs consist of four different subunits with the clockwise arrangement αγαβδ when viewed from the extracellular side. We used disulphide trapping between individually engineered Cys in the αM2 segments to investigate arrangement and flexibility of the upper part of αM2. Disulfide bond formation was monitored in $\alpha_2\beta\delta\gamma$ nAChR expressed in *Xenopus laevis* oocytes by two electrode voltage clamp experiments and Western blotting. In properly arranged Cys pairs disulfide bond formation can either occur spontaneously or it can be induced by oxidizing with copper phenanthroline (CuPhen). Cystine bond formation is reversible by reducing with dithiothreitol (DTT). To eliminate interference from the native vicinal disulphide present in the ligand binding site of the α-subunit, we utilized the background mutations αC192S-C193S. Position αE262C that was previously shown to face the channel formed DTT reducible dimers both spontaneously and upon application of the oxidizing agent CuPhen. Based on Unwin's 4-Å resolution model, the formation of disulfide bonds at this channel level would require substantial movement of the channel-lining M2 segments. No dimer formation was observed in αL263C. We are investigating a series of αM2 Cys mutants to determine which positions in M2 can form disulfide bonds.

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Contribution of Phosphorylation Residues in the A4 Subunit to the α4β2 Neuronal Nachr Function and Expression

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The α4β2 nicotinic acetylcholine receptor (nAChR) is the most highly expressed subtype in the brain and plays an important role in nicotine addiction. Nicotine exerts its action on the brain, mainly on this subtype. Chronic nicotine exposure up-regulates the α4β2 nAChR, increasing the number of receptors in the CNS. The working hypothesis of this study is that the nicotine-induced up-regulation of α4β2 nAChRs may involve subunit phosphorylation. There are thirteen sites in the cytoplasmic loop of the α4 subunit, known to be consensus sites for various protein kinases. Eleven of these sites were mutated, to aspartic acid, to mimic the effect of subunit phosphorylation and to alanine to abolish a potential phosphorylation. To asses receptor function two-electrode voltage clamp was done on the mutated receptors expressed in *Xenopus laevis* oocytes. Binding assays using [^{125}I]-Epibatidine was used to verify the expression of the mutated receptors. Several positions resulted in an apparent ''on/off'' switch for constitutive function; the ''on'' switch being the alanine mutation and the ''off'' switch being the aspartic acid substitution. For example, two alanine mutants, which are PKC phosphorylation residues, resulted in functional receptors with a macroscopic current and expression similar or greater than that of the wild-type α4β2 nAChR. On the other hand the aspartic acid mutants displayed expression levels similar to wild-type but exhibited a dramatic decrease in macroscopic current. These results suggest that these consensus phosphorylation sites can regulate the α4β2 nAChR functional responses without affecting expression. On the other hand, the increase in the EC_{50} for ACh displayed by several mutations is consistent with the functional parameters of nicotine-induced up-regulated α4β2 nAChRs. This suggests that phosphorylation of the α4 subunit may regulate the α4β2 nAChR function and expression.

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M2 Segment Accessibility in The Prokaryotic Proton-Gated Cys-loop Receptor Channel from Gloeobacter Violaceus in Closed and Open States

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The prokaryotic homopentameric proton-gated cation channel from *Gloeobacter violaceus* (Glic) is a putative homologue of the Cys-loop family of neurotransmitter-gated channels, with an ~200 residue N-terminal extracellular domain and 4 transmembrane α-helices per subunit. Glic lacks the large cytoplasmic loop between M3 and M4 and the signature disulfide linkage. High resolution crystal structures of Glic were recently published. To determine whether the Glic structure is a good model for eukaryotic Cys-loop receptors, we tested the accessibility of 30 individual cysteine substitutions in M2 and the M2-M3 loop of Glic to *p*-chloromercuribenzenesulfonate ($pCMBS^-$) in the closed state (pH 7.5) or in a sub-maximally activated state (pH 5). In 11 of the 30 Cys mutants the proton-induced currents were not significantly different than those in water-injected oocytes. Of the mutants tested from −2′ to 7′, E221C (−2′) and T225C (2′) were reactive at both pH 7.5 and 5.0, V224C (1′) and T230C (7′) were reactive only at pH 5.0, and A222C (−1′) was reactive only at pH 7.5. Reactions occurred at rates $<100\ M^{-1}s^{-1}$. The 8′ and 9′ mutants showed aberrant gating properties. From 10′ to 27′ $pCMBS^-$ reacted with all residues except H234C (11′), V241C (18′), T243C (20′) and L245C (22′). Reaction rates at residues more extracellular than 13′ were $>1000\ M^{-1}s^{-1}$. While a clear α-helical pattern is not apparent, given recently published X-ray crystallographic data we infer that M2 is tightly associated with the adjacent transmembrane helices at the intracellular end but is loosely packed from 10′ to the extracellular end. The chemical accessibility data suggest a more loosely packed structure than the crystal structures, which may represent a desensitized state.

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Estimation of Phi-Values for the Am1 Domain of Neuromuscular Acetylcholine Receptors

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The AChR forward 'gating' isomerization occurs as a conformational cascade that starts at the transmitter binding sites and propagates to the gate region. This pattern has been deduced from the progressive reduction in a experimental parameter called Φ. In two the α-subunit transmembrane domains (TMDs), Φ-values are consistent for the membrane-facing residues of M4 (Φ~0.54) and M3 (Φ~0.3), as expected from a rigid body gating motion. Most αM2 residues (17′-1′) have a Φ~0.64, but there are low-Φ side chains near the gate region (12′-9′; Φ~0.3) and high-Φ ones at the N-terminal 'cap domain. (27′-18′; Φ~0.9). Towards the completion of the map of Φ in the α subunit, we are mapping values for the αM1 domain. Here we present preliminary results on diliganded isomerization equilibrium constant (E_2) consequent to mutations of αM1 residues (HEK, mouse $(\alpha1)_2\beta\delta\epsilon$ subunits transfected, 23 °C, −100 mV, cell-attached single-channel recording, 20 mM Cho or 500 ∈ μM ACh). Mutations (n=2-6) at each of the following positions altered E_2 by <-fold (L212, Y213, I219, I220, C222, L223, F227, T229, S230, V232, F233, D238 and S239). Substitutions that increased/decreased E_2 by >5-fold were at positions: F214, I215, V216, N217, V218, L224, F225, S226 and L228. Single-channel currents were observed for mutations at position P221 (to A, C, F, G, R, S, T, V, and Y) although the resulting kinetic behavior was complex. The largest effect in terms of fold-change in E_2 observed so far was at position 228 (L-to-A, ~300-fold, 3.4 kcal/mol). Overall, the energetic consequences of the mutations examined so far in αM1 are moderate compared to the other α subunit TMD helices.

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Photoaffinity Labeling the Agonist Binding Sites of *Torpedo* and α4β2 Nicotinic Acetylcholine Receptors and Acetylcholine Binding Proteins (AChBPs) with [^{3}H]Cytisine

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The subunit composition of brain nicotinic acetylcholine receptors (nAChR) dictates their different physiological, pharmacological and pathophysiological properties. While targeting brain nAChRs is a promising strategy in the treatment of different neurological conditions including Alzheimer's and Parkinson's disease and nicotine dependence, the development of nAChR subtype-selective agents remains a challenge. The partial agonist cytisine and its derivative varenicline (CHANTIX; FDA approved drug for smoking cessation) are examples of drugs with higher selectivity for the α4β2 nAChR subtype. Here, we use radioligand binding, photoaffinity labeling and computational analysis to study the mode of interaction of cytisine with a diverse group of acetylcholine binding sites [nAChRs and ACh-binding proteins (AChBP)]. [^{3}H]Cytisine binds with high affinity (1.6 nM) to α4β2 nAChRs, with low affinity (1.3 ∈ μM) at both α-γ and α-δ agonist binding sites of the *Torpedo* (muscle-type) nAChR and to *L*-AChBP, *A*-AChBP and *A*-AChBP(Y55W) with low to modest affinity, 0.37 ∈ μM, 2.5 ∈ μM, and 80 nM, respectively. Upon UV irradiation, [^{3}H]Cytisine photoincorporated selectively into the α- and γ-subunits of *Torpedo* nAChR . The sites of [^{3}H]Cytisine labeling were determined in each

subunit, αCys$^{192/193}$,αTyr^{198} (Loop C), γTrp^{55} (Loop D) and γTyr^{117} (Loop E) of the agonist binding site. [^{3}H]Cytisine efficiently photolabels the agonist binding sites of AChBPs and the α4β2 nAChR (both α4 and β2 subunits are labeled). The sites of [^{3}H]Cytisine labeling in *the Torpedo* nAChR and in AChBPs and the α4β2 nAChR (ongoing experiments), along with results from cytisine docking simulations will be used to compare modes of interaction of α4β2 nAChR-selective and subtype non-selective agonists (e.g. ACh) to nAChRs and AChBPs.

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Mutations at Ag153 in Nicotinic Acetylcholine Receptors increase the Un-Liganded Gating Equilibrium Constant

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Rarely, an AChR mutation will increase the affinity of the resting receptor for ACh (reduced K_d). One such example is αG153S, a cause of slow-channel congenital myasthenic syndrome. The presence K vs. G side chain at this position in neuronal α4β2 vs. neuromuscular $(\alpha1)_2\beta\delta\epsilon$ AChRs has been suggested to be the basis for the high affinity of nicotine only to the former. In the present study we examined AChRs having a side chain substitution at αG153(ACDKPRSWY). We measured (single-channels, $(\alpha1)_2\beta\delta\epsilon$; −100 mV, 23 °C, cell-attached) the equilibrium dissociation constant (K_d) for the partial agonist choline for the ADRSK mutants, and the diliganded (E_2) and un-liganded (E_0) isomerization ('gating') equilibrium constants for all mutants. The αG153S mutant (wild-type) parameters were: E_2~0.38 (0.05), E_0~3.5E10-6 (1.2E-7), K_d~540 ∈ μM (4 mM) and J_d ~1.64 ∈ μM (6 ∈ μM). The fold-changes in each of these parameters were: E_2, 7.6; E_0 29; K_d, 7.4; and J_d, 3.7. The largest effect of the S substitution was on E_0. Preliminary results for the other 153 mutants also show even larger changes in E_0. The G153K mutation showed a ~210-fold increase in E_0. It is possible that the above effect of the G153K mutation with regard to nicotine activation is due, al least in part, to the increase in E_0. This alone would reduce the EC_{50} of macroscopic currents and effectively change the appearance of nicotine, from a weak to a strong agonist.

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A Transmembrane Binding Site at a Subunit Interface for *Torpedo* Nicotinic Acetylcholine Receptor Potentiators And Inhibitors

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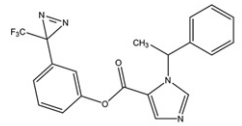
Photoreactive derivatives of the general anesthetic etomidate have been developed to identify their binding sites in $GABA_AR$ and nAChRs. One such drug, [^{3}H]TDBzl-etomidate, acts as a positive allosteric potentiator of *Torpedo* nAChR and binds to a novel site in the transmembrane domain at the γ-α subunit interface (*Nirthanan et al 2008, JBC 283:22053-62*). To extend our understanding of the binding site(s) of nAChR allosteric modulators, we developed [^{3}H]TFD-etomidate containing the photoactivatable trifluromethyldiazirinyl group on etomidate's benzene ring. [^{3}H]TFD-etomidate inhibited ACh-induced currents (IC_{50} = 4 ∈ μM), but inhibited the binding of [^{3}H]phencyclidine to the *Torpedo* nAChR ion channel with IC_{50}s of 2.5 and 0.7 mM in the resting and desensitized states, respectively. In the presence of the ion channel blocker tetracaine, [^{3}H]TFD-etomidate photolabeled amino acids at the lipid interface (αM4 and βM4) and at the γ-α subunit interface, αM2-10, γMet299 and γMet295. In the absence and presence of agonist, [^{3}H]TFD-etomidate photoincorporated at low efficiency within M2 ion channel domain (M2-6, M2-9 and M2-13). These results suggest that the γ-α subunit interface is a binding site for *Torpedo* nAChR negative ([^{3}H]TFD-etomidate) and positive ([^{3}H]TDBzl-etomidate) allosteric modulators.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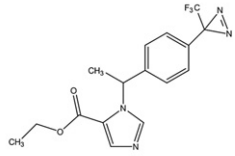
TFD-Etomidate

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Dynamics of Acetylcholine Receptor-Channel Gating: Pre-M1 of the Epsilon Subunit

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Neuromuscular acetylcholine receptors (AChRs) mediate fast chemical synaptic transmission. Neurotransmitters bind to two sites in the α subunit extracellular domain (ECD) and trigger an isomerization that opens/closes the pore in the transmembrane domain (TMD). The TMD/ECD interface is a complex and important domain that links 'binding' and 'gating'. We have examined one component of this interface, the pre-M1 region (linker connecting β10-strand of the ECD to M1 of the TMD) in both α and non-α subunits. We recorded single-channel currents (mouse $\alpha_2\beta\delta\epsilon$; HEK cells, −100mV, cell-attached) and estimated gating rate constants and rate-equilibrium relationships for AChRs having a mutation at the pre-M1 linker of the ϵ subunit. This region is a stretch of five amino acids (217-221) that contains three positively charged residues (ϵR217, ϵR218, ϵK219). ϵR218 is homologous to αR209 and is conserved among other AChR subunits. AChRs were activated by 20mM choline or 0.5mM acetylcholine. So far we have measured the gating rate constants of ϵ subunit mutants at positions R217, R218, and K219. R217D and N decreased the diliganded equilibrium isomerization constant (E_2) by only 1.5-fold. R218A and N decreased E_2 by 184-fold and 235-fold, respectively. K219A, D and N increased E_2 by <6-fold. So far, out of the three scanned positions, R217 and K219 show moderate energy changes (~1 kcal/mol) and R218 shows a larger energy sensitivity (~3.2 kcal/mol). In all cases, both the forward and backward rate constants changed with the mutation, with the larger effect being on the forward rate constant. The results indicate that ϵ pre-M1 changes energy ('moves') during gating isomerization. More experiments should reveal more precisely both the energy sensitivity of each position and the relative timing of the side chain motions within the AChR isomerization. Supported by NIH (NS-23513, NS-064969).

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Potential Implications of Cholesterol and Phosphatidylinositol 4,5-Bisphosphate (PIP2) Interactions With The Cholesterol-Sensitive AC418W Acetylcholine Receptor Mutation at Lipid Rafts

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Lipid rafts, specialized membrane microdomains in the plasma membrane that are rich in cholesterol and sphingolipids, are hot-spots for a number of important cellular processes. The novel acetylcholine receptor (AChR) mutation αC418W was shown to be cholesterol-sensitive (Santiago et al., 2001) and to accumulate in microdomains rich in the membrane raft marker protein caveolin-1 (Baez-Pagan et al., 2008). The objective of this study is to gain insight into the mechanism by which lateral segregation into specialized raft membrane microdomains regulates the activatable pool of AChRs. We performed Fluorescent Recovery After Photobleaching (FRAP) experiments and whole-cell patch clamp recordings of GFP-encoding *mus musculus* AChRs transfected into HEK 293 cells to assess the role of cholesterol levels in the diffusion and functionality of the AChR (WT and αC418W). Our findings support the hypothesis that a cholesterol-sensitive AChR might reside in a specialized membrane microdomain; however, when cholesterol is depleted in vitro or in vivo, the caveolae disrupt and the cholesterol-sensitive AChRs are released to the pool of activatable receptors. Furthermore, our results suggest that phosphatidylinositol 4,5-bisphosphate (PIP_2), which is concentrated in lipid rafts, may be responsible for the increase in whole-cell currents observed upon cholesterol depletion for the αC418W AChR mutant.

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The N Terminal M2 Cap of Nicotinic Acetylcholine Receptors

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Nicotinic acetylcholine receptors (AChRs) isomerize ('gate') between a low affinity/non-conducting (R) and a high affinity/ion-conducting (R*) conformation. Many different residues in this large, heteropentameric membrane protein have been shown to contribute to the free energy difference between the R and R* structures. Previously we showed that at the N-terminal 'cap' of the M2, pore-lining helix (positions 18′-27′) there are large and early energy changes in the α subunit whereas in the ϵ subunit the residues are mostly iso-energetic. We measured the energy sensitivity (computed from the apparent range in diliganded equilibrium constant E_2) and relative timing (Φ values) of the isomerization movements of residues in the N-terminal 'cap' domain of the δ and β subunits. We used cell attached, single-channel analysis to quantify the energetic consequences of point mutations (mouse $\alpha_2\beta\delta\epsilon$, HEK 293 cells, +70 mV pipette potential, cell-attached, 22° C , activated by 20 mM choline or 0.5 mM ACh). The probed positions were δ: I18′, S19′, K20′, R21′, L22′, P23′, A24′, T25′ and M27′ (a total of 43 mutants) and β:A19′, K21′, V22′, P23′, E24′, S26′, and L27′ (a total of 34 mutants). Of these, only one position, δS19′, showed a >2 kcal/mol range-energy (Φ = 0.20 ± 0.07). The only residues