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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