desensitization, while the type 2 PAMs enhance the current amplitude but also slow the desensitization of the receptor. In order to understand the mechanism of action of the type 1 and type 2 PAM's, we tested three modulators on the QPatch automated patch-clamp system using GH4C1 cells stably expressing the rat α-7 receptor. We also explored the effect of repeated applications of these agents on their modulatory activity. Consistent with previously published data, PNU-120596 showed type 2 PAM activity accompanied with a decreasing magnitude of potentiation with repeated applications. Estimated EC50 values for PNU-120596 were stable with repeated compound application. NS-1738 produced a type 1 PAM activity, and a cumulatively increased potentiation following repeated applications, accompanied by an approximate 3-fold increase in EC50. In contrast, SB-206553 had similar potency and effects on the potentiation with repeated application. These results indicate that the different α-7 receptor PAMs have different rates of activation and desensitization, in addition to their type 1 or 2 effects on receptor desensitization.

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Channel Blocking Properties Of Tetramethylammonium At The Human Muscle Acetylcholine Receptor

Remigijus Lape, David Colquhoun, Lucia Sivilotti.
UCL, London, United Kingdom.

Agonists at muscle acetylcholine receptors (AChR) all seem to be able to block the channel as well as activate it. In particular, many partial agonists block the channel pore at concentrations close to those that activate it, e.g. choline or tetramethylammonium (TMA). We recorded TMA-activated single- channel currents in the cell-attached configuration from HEK293 cells expressing human adult AChR. The amplitude of openings recorded at -80 mV appears to decrease progressively with agonist concentration because of fast channel block. The equilibrium constant, K_B, for open channel block was about 9 mM as estimated from the reduction of apparent single channel amplitude. This is comparable with the EC_{50} of 2 mM. Several records obtained at different TMA concentrations were fitted simultaneously with the HJCfit program[1]. Because the blockages were undetectable, the open state and the open-blocked state were treated as a single compound open state in the analysis. In the first instance fits were done with a mechanism that allows block of channels only when they are open. The predicted distribution of apparent open times at the lower concentrations TMA matched the observations quite well, but at the higher concentration the prediction was poor (the predominant mean apparent open time was about 1.5 times smaller than predicted). Then fits were done of a mechanism in which the block is not selective for the open state, but can occur from any state. In this case the distributions of apparent open times were predicted accurately at both low and high concentrations of TMA. The present data suggest that TMA does not act as a pure open channel blocker, but AChRs blocked by TMA can close and return to the resting state without re-opening.

1. Colquhoun *et al. J Physiol* 547, 699, 2003.

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Temperature Dependence And Activation Energy of nAChR Gating

Shaweta Gupta, Anthony Auerbach.
University at Buffalo, Buffalo, NY, USA.

Neuromuscular acetylcholine receptors (AChRs) are ion channels that alternately adopt conformations that either allow or prohibit the flow of ions across the membrane. These two stable end states are separated by an energy barrier, the peak of which is called the transition region. The energy of the transition region relative to the end states is the reaction activation energy (E_{act}). To quantify E_{act}, we studied the temperature dependence of single-channel gating rate constants (k_o, opening; k_c, closing) for wt and mutant AChRs, activated by different ligands or without any added ligand, over a range of temperatures (5-35 ^oC, HEK cells, cell attached, -110 mV, mouse $\alpha_2\beta\delta\epsilon$). The results were fitted by the Arrhenius equation: $k(T)=A*\exp(-E_{act}/RT)$. Increasing the temperature from 5^0 C to 35^0 C increased k_c for wt and δL265T AChRs activated by choline, each by ~35-fold (E_{act}=20.5 and 23.6 kcal/mol, respectively). We also estimated the temperature sensitivity of k_o and k_c in four more constructs with one or more point mutations in both α subunits. For all four, k_o and k_c increased with temperature: E_{act} (k_o and k_c; kcal/mol)=21 and 23 (Y127E activated by ACh); 24 and 27 (D97A + Y127F + S269I, unliganded); 29 and 23 (D97A + Y127F + S269I + W149F, unliganded); 29 and 25 (G153S activated by choline). For these six constructs the average activation energy was ~24.6 kcal/mol for both closing and opening. This quantity did not change with the agonist (including water) or the mutations. This suggests that the energy barrier for the gating isomerization is not significantly determined by the ligands at the transmitter binding sites or by the gating motions of the mutated residues, and that unliganded and diliganded AChR gating likely proceed by similar reaction pathways.

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Interaction Between Two Domains in the AChR Gating Reaction

David J. Cadugan[1], Lin Chen[2], Anthony Auerbach[1].
[1]SUNY at Buffalo, Buffalo, NY, USA, [2]University of Southern California, Los Angeles, CA, USA.

The AChR is a large ion channel that isomerizes between non-conducting and conducting conformations. Residue αA96 is located in loop 5 (loop A) near the agonist-binding site, which moves at the outset of the channel-opening process (the Φ value for the adjacent residue αD97 is 0.93). Side chain substitutions at nearby (<0.45 nm, 2QC1.pdb) residue αY127 (β-strand 6) change the gating equilibrium constant (K_{eq}) by up to 290,000-fold (Φ=0.77). This suggests that αY127 moves in concert with the lower part of the extracellular domain, after the motion of loop 5. αD97 and αY127 are not coupled energetically. We tested the hypothesis that αA96 and αY127 energetically link the first two Φ-blocks, to propagate the opening conformational wave from the upper to the lower part of the extracellular domain. We mutated residue αA96 (C, F, K, L, N, Q) and measured single-channel gating kinetics (mouse $\alpha_2\beta\delta\epsilon$, cell-attached, -100 mV, 20 mM choline, PBS, 23°C). The Φ-value for αA96 is 0.90, indicating that it moves at the onset of channel gating along with other residues in loop 5. αA96N showed the largest change in K_{eq} (~900-fold) and markedly increased unliganded gating. Next, we performed mutant cycle analysis to test for energetic coupling between αA96 and αY127. K_{eq} for the double mutant αA96K+αY127E is 18-fold *greater* than the wt, where the effects of the single mutants, if additive, predict one that is 9.2-fold *smaller*. This corresponds to a coupling free energy of -3.1 kcal/mol. Similarly, K_{eq} for the double mutant αA96C+αY127C is 213-fold *greater* than the wt, whereas a value 1.7-fold *smaller* is predicted assuming independence (coupling free energy of -3.6 kcal/mol). These are large interaction energies that suggest αA96 and αY127 form a key energetic link between the first and second Φ-blocks.

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βM2 of The Neuromuscular AChR: Gating, Desensitization and Orientation

Snehal V. Jadey, Prasad Purohit, Anthony Auerbach.
State University at New York at Buffalo, Buffalo, NY, USA.

The M2 helix of each of the five acetylcholine receptor (AChR) subunit forms the narrow region of the ion conduction pathway. As part of an overall project of trying to understand the mechanisms that underlie two reactions - the C(losed)↔O(pen) conformational change ('gating') and desensitization - we studied single-channel currents from AChRs with a point mutation in the β subunit (mouse $\alpha_2\beta\delta\epsilon$, HEK cells, cell-attached, -100 mV, 23^{o}C, activated by 30 μM ACh). From measurements of cluster open probabilities (P_o) and durations (τ) we could make *qualitative* inferences about the effects of the mutations on gating (P_o; increase, decrease, no effect) and desensitization ($\tau_{cluster}$; altered, no effect). So far, 58 different mutations of 14 different βM2 residues have been examined. For some of these we also quantified the single-channel current amplitude of the R substitution (i_R; small, no effect). The results are as follows. 1) P_o (by mutation): 26 increased, 13 decreased, 18 no effect. The increases were most apparent in the equatorial 9′-12′ region. 2) $\tau_{cluster}$ (by residue): 6 altered and 8 no effect. The altered bursts were mostly prolonged, with the effects being largest at 9′-12′ and 14′-15′. 3) i_R (by residue): 5 small (8′-10′, 13′, 15′), 3 no effect (6 positions not tested). By examining mutants of all βM2 positions, using a saturating concentration of either choline or ACh, we hope to build maps of the energetic consequences with regard to gating and desensitization, and learn the orientation of residues in the Open conformation of the protein.

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The Unliganded Gating Mechanism Of Nicotinic Acetylcholine Receptors

Prasad G. Purohit, Anthony Auerbach.
Suny Buffalo, Buffalo, NY, USA.

The nicotinic acetylcholine receptor (AChR) switches between C (low agonist affinity and low conductance) and O (high agonist affinity and high conductance) conformations ('gating'). The probability of channel opening is very low in the absence of agonist, but when agonists are present at the two transmitter-binding-sites opening increases rapidly (~20μs), transiently to a high probability (~0.95). We observe that 'gain-of-function' mutants that increase the diliganded gating equilibrium constant (without affecting agonist binding to C) also increase the frequency of spontaneous openings. Unliganded openings occur in clusters in AChRs having several of such mutations. We analyzed the intra-cluster interval durations to estimate that the unliganded gating equilibrium constant is $\sim 1.15 \times 10^{-7}$ (mouse, $\alpha_2\beta\delta\epsilon$, -100 mV). The agonist affinity ratios (C vs. O) for acetylcholine, carbamylcholine, tetramethylammonium and choline are ~15,600, ~6700, ~6700 and ~600. The monoliganded (with ACh) gating equilibrium constant is $\sim 1.7 \times 10^{-3}$. Acetylcholine provides only ~0.9 k_BT more binding energy per site than tetramethylammonium, but ~3.1 k_BT more than choline. Mutations of binding site residue αW149 *increase*