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Multiple Modes of Interaction between the Nicotinic Acetylcholine Receptor and Isoflurane Observed Through Long Time Simulations
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Understanding of the molecular mechanisms through which anesthetics affect function of ligand gated ion channels (LGIC) like the nicotinic acetylcholine receptor (nAChR) is complicated by the multitude of binding sites and possible mechanisms that are consistent with experimental data, including pore-block, competitive binding, and allosteric mechanisms. Molecular dynamics (MD) studies of general anesthetics and Cys-loop receptors have been hindered by low resolution and unstable structures and the computational cost of simulating large systems for the long times required to observe all binding modes. We present long time (400 ns) simulations in which isoflurane introduced into the water surrounding the nAChR proceeded to bind to four main classes of site on the protein (as well as partition into the membrane) : 1) the hydrophobic constriction of the channel lumen, effectively blocking the pore 2) the agonist site 3) the interface between subunits, in the transmembrane domain, 4) in the center of some subunits, in the transmembrane domain on the intracellular half. Sites 1) and 2) are most likely inhibitory sites that may not be present in anionic cys-loop channels (which are potentiated by anesthetics);we find analogous sites in a 200 ns simulation of the prokaryotic cationic LGIC from Gloeobacter violaceus (for which two high resolution structures are available). Isoflurane bound to site 3) is found to have distinct and statistically significant effects on the structure and dynamics of the adjacent critical M2-M3 loop, with repercussions in the pore-lining M2 helices,consistent with an allosteric potentiating mechanism that may account for the reverse effect of isoflurane on the GABA and glycine receptors.

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NMR of Transmembrane and Intracellular Domains of Human Nicotine Acetylcholine Receptor A7 Subunit
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Structure determination of mammalian membrane proteins is challenging due to low expression level, unpredictable folding, and poor protein stability. We addressed these critical issues using a novel approach to break hydrophobic patches by minimal mutagenesis in transmembrane (TM) domains. For a 300-residue sequence encompassing the entire TM and long intracellular (IC) loop domains of the human nicotinic acetylcholine receptor (nAChR) α7 subunit, 16 mutation sites were identified and experimentally implemented. The resultant mutant (nAChRα7TM-IC) was expressed with a typical yield of 5 mg/L in M9 medium for NMR investigation. Replacing IC loop with a 5-Gly linker (nAChRα7TM-5G) increased the yield to 10 mg/L in M9. Both nAChRα7TM-IC and nAChRα7TM-5G folded into stable structures in ~1% empigen with helical content of 41% and 50%, respectively, determined by circular dichroism. Thus, the helical content of IC can be calculated to be 29% at least, in agreement with sequence-based predictions. Exceptionally high-resolution and well-dispersed NMR spectra, comparable to those of soluble proteins of similar size, were obtained for nAChRα7TM-5G, allowing for spectral assignment and structure calculation. Reasonably high-resolution NMR spectra of nAChRα7TM-IC were also acquired at 900 MHz, with majority of TM-domain peaks overlapping with those in nAChRα7TM-5G, suggesting that the presence of IC domain did not greatly change the structure of TM domains. Backbone dynamics analyses of the two proteins using our newly developed histogram method, which does not require full spectral assignment, showed that the presence of IC dramatically affected the intrinsic dynamics of TM domains. Details of NMR structure characterization of TM and IC domains of nAChR will be presented. The approach is readily applicable to other receptor proteins, opening a new avenue for structural investigation of membrane proteins. Supported by NIH (R37GM049202, R01GM056257, R01GM069766, and P01GM055876).

Channel Regulation & Modulation I

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Ca^{2+} Transients In Pim-1 Transfected Cardiac Stem Cells Co-Cultured With Rat Neonatal Cardiomyocytes
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Cardiac stem cells transfected with serine/threonine kinase (Pim-1 cells) have been demonstrated to enhance the regenerative capacity upon adoptive transfer to infracted recipient mice (Fischer, *et al.*, 2009, circulation). To probe the possible mechanisms at play in such hearts, we investigated the electrophysiological and Ca^{2+} signaling events in Pim-1 cells co-cultured for 3 days with ventricular myocytes of 4-6 day old rats. Membrane capacitance of whole-cell voltage-clamped control Pim-1 cells (33 ± 3 pF, n=25) was smaller compared to cardiomyocytes (51 ± 10pF, n=10), but increased significantly to 164 ± 16pF (n=8) in co-cultured Pim-1 cells. Co-cultured Pim-1 cells developed I_{Ca} that was smaller (0.63 ± 0.22pA/pF) and activated more slowly (16 ± 2ms time to peak) than in cardiomyocytes (3.32 ± 0.69pA/pF; 5.0 ± 0.3ms). Confocal Ca^{2+} imaging (Fluo-4AM) showed strong Ca^{2+}-release signals in cultured myocytes in response to KCl-depolarization ($\in''F/F_0$= 0.86 ± 0.20, n=11) or 10 mM caffeine puffs ($\in''F/F_0$= 1.42 ± 0.25, n=11), but not in co-cultured Pim-1 cells ($\in''F/F_0$= 0.13 ± 0.03, n=3 and 0.15 ± 0.03, n=6, respectively). However, Pim-1 cells produced large, slowly decaying Ca^{2+}-signals ($\in''F/F_0$= 1.17 ± 0.16, n=19) on rapid application of 100 ∈ μM ATP. In contrast, cardiomyocytes generally responded weakly to ATP applications ($\in''F/F_0$= 0.35 ± 0.12, n=4). The expression of significant I_{Ca} in Pim-1 cells in 3 day old co-cultures suggests *de novo* expression of Ca^{2+} channels, but the increase in membrane capacitance and the delayed activation of I_{Ca} in these cells may also be consistent with development of gap junctions between cardiac myocytes and Pim-1 cells. The finding of IP_3-gated Ca^{2+} stores in the Pim-1 cells, in a manner similar to embryonic or neonatal cardiomyocytes, suggests early cardiac differentiation.

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Calmodulin-Like Region of $Ca_V1.3$ Harbors Novel Structural Determinants Underlying CaM-Mediated Channel Regulation
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The regulation of Ca_V channels by calmodulin (CaM) is both biologically critical and mechanistically rich, rendering this system a central prototype for ion-channel modulation. Despite a decade of study, little is known of the structural mechanisms underlying such modulation, beyond the initial preassociation of Ca^{2+}-free CaM (apoCaM) with an IQ domain on the carboxy-terminus of channels. We have recently argued that the ultimate end-point of channel regulation is allosteric modulation of S6 cytoplasmic gates (*Biophys J*96:222a). However, the transduction events®'those linking calcification of apoCaM to this allosteric effect®'remain essentially unknown. The majority of structure-function analyses have narrowly focused on the IQ domain and immediately upstream 'preIQ' regions, despite hints that further upstream elements in the carboxy-terminus could be important. Additionally, deletions and non-conservative mutations have often been employed in these analyses, confounding interpretation with the potential for backbone fold disruption. Here, we undertook exhaustive alanine scanning mutagenesis of the carboxy-terminus, up to the IQ domain of $Ca_V1.3$ channels. Importantly, the substitution of alanines likely preserves backbone fold throughout. Moreover, $Ca_V1.3$ (highly homologous to classic $Ca_V1.2$ channels) exhibits robust CaM-mediated inactivation (CDI) that enhances structure-function analysis. Surprisingly, alanine substitutions throughout the preIQ domain left CDI essentially unchanged, at odds with functional hotspots in the homologous region of $Ca_V1.2$ (^{1582}NEE^{1585}, ^{1572}IKTEG^{1576}, and ^{1600}LLDQV^{1605}). Instead, newly identified and critical segments were situated upstream, in a region predicted to resemble a lobe of CaM by structural modeling (*Rosetta*). Intriguingly, homologous residues of Na_V channels are linked to mutations underlying heritable LQT syndromes, hinting at conserved modulatory mechanisms across Na_V and Ca_V channels. Overall, this alanine scan of the $Ca_V1.3$, together with that of the IQ domain in a companion abstract, lays the groundwork for understanding the structure-function mechanisms underlying CaM/channel regulation.

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Apocynin Reversibly Inhibits L-type Ca^{2+} Channel Current Involvement of Reactive Oxygen Species
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L-type Ca^{2+} channel current ($I_{Ca,L}$) is a major Ca^{2+} entry mechanism and contraction of arterial smooth muscle (ASM) is regulated by reactive oxygen species (ROS). We utilized apocynin (APO) as a tool to clarify the contribution of ROS in the regulation of $I_{Ca,L}$. APO, a natural organic compound contained in a variety of plants, is widely used as inhibitor of NADPH oxidase (NOX) that reduces oxygen to superoxide in the presence of NADPH to generate ROS including H_2O_2. We recorded whole cell $I_{Ca,L}$ with Ba^{2+} as charge carrier from isolated bovine coronary ASM (BCASM) and HEK293 cells transiently expressing human cardiac CaV1.2. APO was introduced and washed out during