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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 $\in$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

continuous application of depolarization step to 0 mV from HP −80 mV at 1/min. We found that APO inhibited $I_{Ca,L}$ in a dose-dependent manner between 0.1 and 10 mM decreasing its amplitude to ~20% of control. APO also accelerated $I_{Ca,L}$ decay during depolarization. Surprisingly, washout of the high concentration of APO caused rapid recovery of $I_{Ca,L}$. It could even produce a rebound increase of $I_{Ca,L}$ with its peak at ~3 min in BCASM. We performed fluorometric analysis of APO-induced change of cellular ROS by loading cells with 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA). Oxidation of CM-H_2DCF to CM-DCF by ROS induces an increase of fluorescence. APO markedly inhibited the fluorescence increase, while washout of APO caused more intensive increase of fluorescence than control. The results are explained by the inhibition of NOX by APO which results in decrease of ROS and overproduction of ROS during washout utilizing accumulated NADPH produced by prior NOX inhibition. ROS which changes dynamically *in situ,* e.g. hypoxia and reoxygenation, seems to be vital to sustain $I_{Ca,L}$.

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Caveolin-3 Directly Interacts and Regulates the Function of Cardiac $Ca_V3.2$ (A1H) T-Type Ca^{2+} Channels

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Voltage-gated T-type Ca^{2+} channel (TTCC), $Ca_v3.1$ and $Ca_v3.2$, are normally expressed during cardiac development but are re-expressed in cardiac hypertrophy and may contribute to the altered intracellular Ca^{2+} during this disease. However, the mechanism of altered Ca^{2+} signaling in cardiac hypertrophy is not clearly known. Caveolae containing scaffolding protein caveolin-3 (Cav-3), provide spatiotemporal regulation of intracellular Ca^{2+} in cardiomyocytes. To define the source of signaling Ca^{2+} involved and basis of dysregulated contractile function in cardiac hypertrophy and to investigate the role of caveolae and TTCC in the regulation of Ca^{2+} signaling, we used a transthorasic aortic constriction (TAC) induced mouse model of cardiac hypertrophy. Western blot analysis revealed re-expression of $Ca_v3.1$ and $Ca_v3.2$ proteins and significant increase in expression of Cav-3 in adult ventricle from TAC mice but not from sham treated mice. Electron microscopy analysis demonstrated significant increase in the number of caveolae and co-localization of $Ca_v3.2$ and Cav-3 in the ventricular myocytes in the TAC hearts. Co-immunoprecipitation analysis using anti-Cav-3 antibody revealed that re-expressed $Ca_v3.2$ co-IPs with Cav-3 in the TAC hearts, but not in sham hearts. GST pull-down analysis using Cav-3 fusion proteins confirmed that Cav-3 directly associates with Cav3.2 channels. Whole cell patch clamp analysis in HEK293 cells co-expressed with either $Ca_V3.2$ and wild-type Cav-3 or GFP revealed that co-expression of $Ca_v3.2$ + Cav-3 significantly decreased the peak $I_{Cav3.2}$ (−12 ± 3 pA/pF, n=11) compared to $Ca_v3.2$+GFP (−31 ± 4 pA/pF, n=11). Whereas co-expression of Cav-3 had no effect on the $I_{Cav3.1}$. Cav-3 coexpression did affect the voltage dependent activation or inactivation of $I_{Cav3.2}$. We conclude that Cav-3 associates with $Ca_v3.2$ channels and regulates its function. Increased Cav-3 expression may play a crucial role in regulation of Ca^{2+} signaling during hypertrophic cardiomyopathy.

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Modulation of the Cardiac Transient Outward Potassium Current by CaMKII is Dependent on Lipid Rafts Integrity

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The Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) phosphorylates the Kv4.2/Kv4.3 channel and slows down the cardiac I_{to} current inactivation. Thereby the regulation of the I_{to} channel by CaMKII regulates the duration of the plateau phase of the action potential and the calcium entry into the cell. The expression of the CaMKII is very high in the heart, therefore the compartmentalization is essential to get its specificity. We hypothesized that the I_{to} channel forming proteins Kv4.2/Kv4.3 and CaMKII colocalized within the cholesterol enriched membrane microdomains named lipid rafts. We used freshly isolated ventricular myocytes isolated from Sprague-Dawley rats. I_{to} current recordings were made by the Patch-Clamp technique. Membrane rafts were isolated by centrifugation in a discontinuous sucrose density gradient. Protein-protein interactions were determined by co-immunoprecipitation. The different proteins were visualized by western blot. The Kv4.2, Kv4.3 and CaMKII proteins were localized by immunohistochemistry. Pach-Clamp recordings show that cholesterol depleting agent metil-β-cyclodextrine, eliminates the CaMKII effect on I_{to}. This result indicates that the I_{to} channel and CaMKII are localized in lipid rafts. In contrast, when we incubate the cells with colchicine, a microtubule disrupting agent that internalizes caveolae, the CaMKII effect on I_{to} is not modified. Separation in density gradients show that the CaMKII is localized in lipid rafts as well as the Kv4.2/Kv4.3 channels. In the co-immunoprecipitation experiments we observe that CaMKII is pulled down with Kv4.2/Kv4.3, but not with caveolin. Immunocitochemistry experiments show that there are two populations of Kv4.2/Kv4.3 channels. The CaMKII regulates the population localized in non-caveolar lipid rafts, whereas a different population is localized in caveolae and is not regulated by CaMKII. Supported by a MEC grant (SAF2007-61159).

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Rescue of a Trafficking Defective Human Pacemaker Channel Via a Novel Mechanism: Roles of Src, Fyn, Yes Tyrosine Kinases

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Therapeutic strategies such as using channel blockers and reducing culture temperature have been used to rescue some long-QT associated voltage-gated potassium Kv trafficking defective mutant channels. A hyperpolarization-activated cyclic nucleotide-gated HCN4 pacemaker channel mutant (D553N) has been recently found in a patient associated with cardiac arrhythmias including long-QT. D553N showed the defective trafficking to the cell surface, leading to little ionic current expression (loss-of-function). We show in this report that enhanced tyrosine phosphorylation mediated by Src, Fyn, and Yes kinases was able to restore the surface expression of D553N for normal current expression. Src or Yes, but not Fyn, significantly increased the current density and surface expression of D553N. Fyn accelerated the activation kinetics of the rescued D553N. Co-expression of D553N with Yes exhibited the slowest activation kinetics of D553N. Src, Fyn, and Yes significantly enhanced the tyrosine phosphorylation of D553N. A combination of Src, Fyn, and Yes rescued the current expression and the gating of D553N comparable to those of wild-type HCN4. In conclusion, we demonstrate a novel mechanism using three endogenous Src kinases to rescue a trafficking defective HCN4 mutant channel (D553N) by enhancing the tyrosine phosphorylation of the mutant channel protein.

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hERG1 Channels In Cancer Cells: Physical and Functional Interaction With Integrin Receptors

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The expression and activity of different channel types mark and regulate specific stages of cancer progression, from cell proliferation and apoptosis, to invasiveness, angiogenesis and metastatic spread. As is being increasingly recognized, some of these roles can be attributed to signaling mechanisms independent of ion flow. Evidence is particularly extensive for K+ channels. For example, intracellular signaling cascades can be triggered when ion channels form protein complexes with other membrane proteins such as integrins or growth factor receptors.

Work in our lab has established that hERG1 K+ channels are often aberrantly expressed in primary human cancers and exert pleiotropic effects in cancer cells, in turn regulating cell proliferation, cell motility and invasiveness or stimulating the process of neo-angiogenesis. hERG1 can induce such diverse effects since it triggers and modulates intracellular signaling cascades. This role depends on the formation, on the plasma membrane of tumor cells, of macromolecular complexes with integrin receptors, in particular with the β1 subunit. Recent FRET experiments have clearly shown that hERG1 and β1 directly interact, the intermolecular distance between the two proteins being around 4 nm. Moreover, the hERG1 protein inside the complex could function differently from its classical role in excitable cells, i.e. independently of ion flux, but through a conformational coupling with the partner protein(s). On the whole, data gathered so far allow us to propose a novel antineoplastic therapeutical approach, based on the targeting and unlocking of the β1 /hERG1 complex, in order to impair the hERG1-mediated signaling in cancer cells.

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Substance P and Bradykinin Activate Alternative Gq/11-Coupled Signalling Cascades and Impose Opposite Effects on M Current in DRG Neurons

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We investigated signalling cascades and coupling to M channel modulation of two types of $G_{q/11}$-coupled receptors in rat nociceptive DRG neurons: bradykinin (BK) B_2 and substance P (SP) neurokinin (NK) receptors. In patch clamp experiments, BK induced a rapid and reversible inhibition of M current which was prevented by blocking phospholipase C or buffering cytosolic Ca^{2+}. In contrast, SP (1uM) failed to inhibit M current in 35 neurons tested, instead producing slow augmentation (162 ± 18%) in 19/35 predominantly TRPV1-positive neurons. The augmentation was not reversible by washout but was