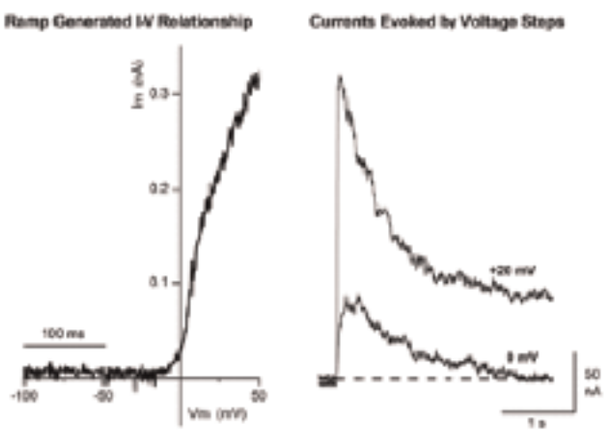
(i.e. immunological disorders, immuno-suppression, metabolic syndrome). Kv1.3 plays a critical role in the activation of human T-cells and are also normally expressed in Jurkat cells, a leukemic cell line well established as an experimental model. In this study perforated-patch whole-cell recordings from Jurkat cells were obtained with IonWorks™ HT and Quattro systems. Composition of the recording internal solution was (in mM): K-gluconate 100, $MgCl_2$ 1, EGTA 5, HEPES 5 (pH= 7.2 with KOH). The external solution was PBS (pH=7.4). Identification of Kv1.3 currents was accomplished by characterizing their kinetical, gating and pharmacological (Margatoxin blockade) properties. In all these respects the results obtained were entirely comparable to Giga-ϑ seal pipette recordings therefore validating the use of automated instrumentation for the electrophysiological study of this channel.



1404-Pos ICh-MASCOT- A Flexible And User Friendly Software For The Global Kinetic Modeling Of Ion Channel Gating

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Board B380

Ion channels in excitable cells play crucial roles in electrical signaling. To gain insight into the gating mechanisms of ion channels, Markov models are commonly used to describe their kinetics quantitatively. Here, we present the Windows-based software "ICh-MASCOT", which is a flexible and user-friendly program for global fitting of ion channel kinetics based on Markov models. The global fit uses experimental data obtained under different conditions to get a set of rate constants that describes the entire experimental data set simultaneously. ICh-MASCOT can use macroscopic data such as time dependence of the current in response to a voltage and/or ligand stimuli, the steady state properties and time constants. Ionic currents for several permanent ions and gating currents can also be included in the global fit. The program supports models for coupled gates described by a single kinetic scheme as well as models with independent gates described by several kinetic schemes. The models can assume arbitrary rate constants with certain voltage and ligand dependencies.

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Acetylcholine Receptors

1405-Pos The Mechanism Of Partial Agonism In The Nicotinic Receptor Superfamily

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Board B381

A partial agonist is a ligand which, at high enough concentrations to occupy all receptors, can elicit only a relatively small response. In the case of ligand-activated ion channels, it has been supposed ever since 1957 that the basis for the small response lies in the gating reaction, i.e. the change of conformation from an open channel to a shut channel. We investigated partial agonists for two members of the C-loop family, namely, tetramethylammonium for the nicotinic acetylcholine receptor and taurine for the glycine receptor. Single channel currents were recorded from HEK293 cells transfected with wild type acetylcholine receptor α, β, ε and δ or glycine receptor α_1 and β subunits in cell-attached configuration. Several mechanisms were fitted by maximising the likelihood of the entire sequence of open and shut time periods, with exact allowance for missed brief events (program HJCFIT[1]). Several records obtained at different agonist concentrations were fitted simultaneously. We found that the results can be well described by a 'flip' mechanism[2] in which after binding, the receptor moves through an intermediate shut conformation ('flip' state), before the channel opens. For both nicotinic and glycine receptors, full and partial agonists showed very similar gating reactions, so differences in gating were not responsible for partial agonism. Rather, the difference between full and partial agonists originated during the earlier conformation change (flipping) while the channel is still shut. This interpretation places the root of partial agonism earlier in the chain of events that follow binding than has been supposed up to now. That is something that might be detectable in structural measurements and could be exploited in rational drug design.

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1406-Pos Interplay between Cations, Anionic Lipids, and Lipid-Protein Interactions at the Nicotinic Acetylcholine Receptor

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Board B382

The role of lipid ionization state in lipid-protein interactions at the nicotinic acetylcholine receptor (nAChR) has been investigated.

Mixtures of phosphatidylcholine (PC) and phosphatidic acid (PA) are particularly effective at stabilizing a functional nAChR. Incorporation of the nAChR into PC/PA 3:2 membranes leads to altered bilayer packing, reflected by a shift in the gel-to-liquid crystal phase transition at pH 7 from ~13 °C (-nAChR) to ~25 °C (+nAChR). To test whether the ability of PA to adopt both mono- and di-anioinic states plays a role in lipid-nAChR interactions, we monitored the pK_a of the mono- to di-anionic transition of PA in membranes with and without the nAChR. In the absence of the nAChR, PA in membranes composed of PA and PC/PA 3:2 exhibits a pK_a for the mono- to di-anionic transition at pH 8.7 and 6.5, respectively. In the presence of the nAChR, however, the PA in PC/PA 3:2 membranes is stabilized exclusively in the mono-anionic state. The gel-to-liquid crystal phase transition temperatures of PC/PA 3:2 lipid membranes with PA in the mono-anionic (pH 4) and di-anionic (pH 10) states are 19 and 2 °C, respectively. Stabilization of mono-anionic PA in PC/PA 3:2 membranes thus accounts for some of the shift in the gel-to-liquid crystal transition temperature upon nAChR incorporation. Increasing concentrations of other cations at the bilayer surface accounts for the remaining shift in gel-to-liquid crystal phase transition in the presence of the nAChR. We conclude that the nAChR, which is a cation selection ion channel, concentrates cations at the membrane surface. The increased local concentration of cations alters local bilayer packing properties.

1407-Pos Closed- and Open-Channel Structures of Neuronal nAChR $(\alpha4)_2(\beta2)_3$

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Board B383

The $(\alpha4)_2(\beta2)_3$ nicotinic acetylcholine receptor (nAChR) is one of the most widely expressed subtype in the brain and shows supersensitivity to volatile anesthetics. The experimental $(\alpha4)_2(\beta2)_3$ structure is unavailable. Using the structure of the *Torpedo* nAChR (PDB: 2BG9) as a template, we built a closed-channel model for the human $(\alpha4)_2(\beta2)_3$. The high sequence identity of α 4 (54%) and β2 (46%) subunits between the target and the template ensured the quality of the model that was further used for generating an open-channel structure based on normal mode analysis. The application of a 'twist' motion eigenvector to the closed-channel elicited a pore opening from R_{min}=2.69Å to 3.41Å. Nicotine docking showed ~38% occupancy in the predicted binding sites in the open-channel structure, while no docking was observed for the closed-channel. During the subsequent 11-ns MD simulations, the open-channel remained open with filled water and a relatively uniform 4.0-Å pore radius; in the closed-channel, the hydrophobic side-chains of V15'-L18' restricted the water passage and made the water density four times less in this region than in the open-channel. Unlike the α4 subunit, each β2 could potentially form two pairs of salt-bridges at the interface between extracellular and transmembrane (TM) domains: R48(β1,2-loop)-D268(TM2,3-linker) and D140(Cys-loop)-K274(TM2,3-linker). In the closed-channel, only the first pair formed stable salt-bridges in two of the β2 subunits. In the open-channel, the salt-bridges between the first pair became unstable but transient salt-bridge between the second pair was formed in one β2 subunit. The formation of the second pair was associated with the tilting of the TM2 helix near the extracellualr interface. This caused the V15' side-chain to rotate away from the center of the pore, implicating a plausible channel gating mechanism.

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1408-Pos The Role Anionic Lipid Head Group Size in Stabilizing a Functional Nicotinic Acetylcholine Receptor

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Board B384

Mixtures of phosphatidylcholine (PC) and the anionic lipid, phosphatidic acid (PA), are effective at stabilizing a functional nAChR whereas mixtures of PC and the anioinic lipid, phosphatidylserine (PS), are not. The enhanced ability of PA to stabilize a functional nAChR is not due to the fact that PA, uniquely amongst natural anionic lipids, adopts both mono- and di-anionic states. To test whether head group size plays a role in the efficacy of PA in stabilizing a functional nAChR, we reconstituted the nAChR into membranes containing PC and three other anionic lipids. Increasing concentrations of phosphatidylglcerol (PG) in PC membranes, were found to increase the proportion of receptors in a functional state, although not with the same efficacy as PA. In contrast, high levels of either phosphatidylinositol (PI) or cardiolipin in PC membranes had no effect upon the ability of the nAChR to undergo agonist-induced conformational change. The ability of the nAChR to influence the packing of lipid bilayers containing PC and either PG, PI, or cardiolipin was also examined. Incorporation of the nAChR into all these membranes increased the lateral packing density of the bilayer, as monitored by a shift up in the gel-to-liquid crystal phase transition temperature and/or a decrease in water penetration into the bilayer polar/non-polar interface, regardless of head group size. Our results suggest that mixtures of PC and anionic lipids are only effective at stabilizing a functional nAChR when the anionic lipid has a relatively small headgroup that alters the physical packing of the PC membranes. In contrast, incorporation of the nAChR into any PC membrane containing anionic lipids alters the packing of the bilayer, possibly by concentrating cations at the bilayer surface.

1409-Pos Cholesterol Modulation Of Acetylcholine Receptor Organization And Stability At The Cell Membrane

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Board B385

The nicotinic acetylcholine receptor (AChR) function and distribution is quite sensitive to cholesterol (Chol) levels in the plasma

membrane (reviewed in Barrantes, 2007). Here we examined the mobility of the AChR and its sensitivity to Chol at the cell surface of CHO-K1/A5 cells, a mammalian cell line that stably expresses adult murine AChR, using the fluorescence recovery after photobleaching (FRAP) technique in the confocal mode. Plasma membrane AChR exhibited limited mobility and only ~55% of the fluorescence recovered within 10 minutes after photobleaching. Depletion of membrane Chol by metyl-beta-cyclodextrin (CDx) strongly reduced the mobility of the AChR at the plasma membrane; the fraction of mobile AChR fell to 20% in Chol-depleted cells. Latrunculin-mediated actin depolymerization restored receptor mobility in Chol-depleted membranes back to control levels. Replenishment of Chol levels with CDx-Chol complexes facilitated receptor mobility at the cell surface. Experimental results and simulation studies suggest that membrane Chol modulates AChR mobility at the plasma membrane through Chol-sensitive receptor (-lipid?) domains synergistically coupled to cytoskeletal integrity.

1410-Pos Single-molecule Dynamics Of Ligand-gated Ion Channels

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Board B386

The rapid signal transduction among neurons and between neurons and e.g. muscle cells is both mediated and modulated by ligand-gated ion channels activated by presynaptically released neurotransmitters. Here, we investigate mobility and functionality the serotonin type 3 and muscle type acetylcholine receptors using fluorescence and electrophysiology methods. The receptor proteins were specifically labeled using fluorophore- or quantum dot-conjugated ligands and NTA-probes, which bind specifically to the binding site and C-terminal oligo-histidine tags, respectively. Next to freely diffusing or immobile receptors, also confined diffusion of the receptors was observed in regions as small as several tens of nanometers. Cytoskeletal scaffold proteins might be implicated in the confinement of receptor proteins. The agonist-induced activation of the receptors affected the diffusional behavior of the receptors.

1411-Pos Gating Dynamics of The AChR Epsilon Subunit M2 Domain

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Board B387

Diliganded acetylcholine receptor-channel (AChR) gating was investigated by measuring the slope (Φ) of rate-equilibrium free energy relationship (REFER), which probes the transition region between C(losed) and O(pen). We think Φ-values reveal the relative timing of residue motion within the overall conformational 'wave'. We used cell-attached, single-channel analysis REFER analysis to quantify the effects of mutations in M2 pore lining domain of the ε-subunit (mouse $\alpha_2\beta\delta\varepsilon$, HEK cells, +70 mV pipette, 22 °C, activated by 20 mM choline or 500 mM ACh). The eM2 helix has 27 residues that run (intra- to extracellular) from C253 to S280. So far we have measured the gating rate constants for 27 eM2 mutants, at positions V255, I257, A262, Q263, F266, E276, T277, S280, and V281. There was no significant effect of any mutation on the apparent channel current amplitude. The side chain substitutions E276 (D), T277 (D, E,Q,R) and S280 (A, D) had little effect and changed diliganded gating equilibrium constant (K_{eq}) by $<$ 3-fold, whereas the other mutations changed K_{eq} $>$10-fold. These substantial changes in K_{eq} arose from changes in both the channel-opening and -closing rate constants, approximately to equal extents. This result suggests that many εM2 residues move during diliganded C↔O, and that this movement in both the upper and lower halves of the segment occurs approximately midway through the reaction. So far, we find that only the previously-probed equatorial residue L261 moves later than the rest of εM2. (NIH NS-23513)

1412-Pos Structure of Affinity-Purified *Torpedo* nAChR revealed by [^{125}I]TID photolabeling

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Board B388

The *Torpedo* nicotinic acetylcholine receptor (nAChR) is the only member of the Cys-loop superfamily of ligand gated ion channels (LGIC) that is available in high abundance in a native membrane preparation. To study the structure of the other LGICs using biochemical/biophysical techniques, in most cases detergent-solubilization, purification, and lipid-reconstitution are required. To test the effects of detergent-solubilization and lipid-reconstitution on the (fine) structure of a LGIC, we solubilized, affinity-purified, reconstituted, and photolabeled the *Torpedo* nAChR with the hydrophobic probe 3-trifluoromethyl-3-(m-[^{125}I] iodophenyl) diazirine ([^{125}I]TID) and compared the pattern of labeling with that observed for the receptor in its native membrane. nAChR-rich *Torpedo* membranes were solubilized in 1% cholate and the nAChR was purified on an acetylcholine affinity column and reconstituted in a lipid mixture of dioleoylphosphatidylcholine, dioleoylphosphatidic acid and cholesterol (molar ratio 3:1:1). The reconstituted *Torpedo* nAChR was labeled with [^{125}I]TID in the absence or presence of agonist. Consistent with that observed for *Torpedo* nAChR in native membrane:

(i) addition of agonist dramatically reduced the efficiency of [^{125}I]TID photoincorporation into M2-9' and M2-13' of each nAChR subunit (reduction: α, 85%; δ, >95%);
(ii) At the lipid-protein interface of the α subunit, [^{125}I]TID labeled αCys^{222} and αPhe^{227} within the M1 segment and αCys^{418} within the M4 segment in an agonist-insensitive manner.

Unlike labeling of *Torpedo* nAChR in native membranes, equilibration with agonist did not enhance [^{125}I]TID photoincorporation within the δM1 (δPhe232) or the δM2-M3 loop (δThr274 and δILe288). These results suggest that purification and reconstitution of *Torpedo* nAChR, under optimal conditions that preserve channel gating, retain the structure of the lipid-protein interface and the change in structure of the M2 ion channel domain associated with desensitization, but not that within the δ subunit intrahelical bundle.

1413-Pos Activation of Homomeric alpha 7 Nicotinic Acetylcholine Receptors

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Board B389

In order to understand the relationship between subunit occupancy and channel opening of homomeric human alpha7 receptors we recorded the mean whole cell macroscopic current response to 0.1, 0.3, 1, 10 mM acetylcholine under voltage clamp using a rapid application system with a 10–90% solution exchange time of 3.5 ms. We fitted these responses to a series of models with 1,2,3,4 or 5 non-cooperative acetylcholine binding steps. The best fits were obtained with models containing 5-binding sites in which the desensitized states either do or do not undergo agonist binding and unbinding. During these fits the channel closing rate was set to 2000 s^{-1} to obtain a mean open time of ~0.5 ms (Fucile et al., 2001). The rate of recovery from desensitization was set to 0.5 s^{-1} to approximate the half-maximal recovery rate described for rat alpha7 receptors (Mike et al., 2000). The K_D for acetylcholine binding to non-desensitized receptors can be calculated from the fitted association and dissociation rates (k+1, k−1), and was 114 micromolar. It is clear from the fitted opening rates for receptors with 1,2,3,4 or 5 agonists bound (1, 1, 47, 778, 585 s^{-1}) that there is little or no receptor opening before 4 or 5 acetylcholine molecules are bound. Similarly, desensitization rates accelerate greatly when 4 or 5 acetylcholine molecules are bound (22, 5, 1, 105, 207 s^{-1}). Thus, P_{open} shows a non-linear dependence on the fraction of agonist binding sites occupied. These results suggest that homomeric human alpha7 receptors will rarely open when occupied with 1–3 molecules of acetylcholine, and must pass through conformations with 1, 2, or 3 agonists bound before the receptor can reach states with higher P_{open}. As predicted from inspection of the rate constants, the P_{open} strongly varies with subunit occupancy.

1414-Pos *T*he effects of AChR mutations at residues εW55 and δW57

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Board B390

The agonist binding sites in the muscle nicotinic acetylcholine receptor (AChR) are located mainly in the α subunit but at an interface with the ε/δ subunit. Two nearby non-α Trp residues, εW55 and δW57, have been shown to play a role in agonist binding and to alter the concentration-response profile. We have examined the effects of mutations of these residues on the rate constants for agonist binding and channel gating (mouse $\alpha_2\beta\delta\varepsilon$, cell attached, +70mV pipette, PBS, 22°C, 30–3000mM ACh). The cluster open probability was reduced by all mutations, in the order ε: I>F>E>C>S>H>A and δ: E>F>V>A>L. Channel-block at high agonist concentrations made the separation of effects on binding vs. gating difficult. Mutation of the ε residue had a greater effect than of the δ residue. Preliminary results suggest that the main effect of the mutations is to reduce the gating equilibrium constant.

1415-Pos Probing Achr Desensitization Using Gating Gain-of-function Mutations

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Board B391

Under continuous application of high concentrations of agonist, acetylcholine receptors (AChRs) adopt high-affinity, non-conducting 'desensitized' (D) conformations. In single-channel patch clamp recordings, such sojourns occur along with 'bursts' that reflect transitions between diliganded-open and -closed conformations. We examined the distributions of D interval lifetimes (mouse $\alpha_2\beta\delta\varepsilon$, HEK cells, cell-attached, PBS, 22° C, 500 mM ACh in the pipette only, +70 mV pipette potential, 10 kHz filter, 25 kHz sampling frequency) to investigate the effect of a subunit mutations known to increase the diliganded gating equilibrium constant on the lifetime distribution of D intervals. D97A, C418W, and L279W each increase the gating equilibrium constants by 168-, 115-, and 156-fold, respectively, and with characteristic phi-values of 0.93, 0.51, and 0.30. We interpret these values to indicate that these three residues move early, in the middle, and late in the gating reaction. In wt AChRs, the non-conducting interval durations were described by either four or five components, and the burst durations (representing both A_2C and A_2O conformations) were described by a single component (Elenes and Auerbach, J Physiol, *541*:367). The non-conducting interval durations for D97A AChRs had four components, with time constants and relative amplitudes that were indistinguishable from the wt. The non-conducting interval durations for C418W and L279W had five components, again unchanged from the wt. For all mutants, the burst durations and single channel amplitudes were also unchanged from the wt. These preliminary results suggest that these mutants, which all had a substantially increased diliganded gating equilibrium constant, are indistinguishable from the wt. Analysis of additional mutations and different agonists may allow us to further understand AChR desensitization.

1416-Pos Examining the Relationship Between γW55/δW57 and Pore Mutations in the nAChR- Analysis of a Two-Component Dose-Response Relation

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Board B392

Nicotinic acetylcholine receptors (nAChRs) are members of the Cys-loop receptor family and mediate synaptic transmission at the neuromuscular junction and in the CNS. Ligand binding in the extracellular domain favors the opening of a cation-conducting channel, the gate of which is ~ 60 Å away. While a considerable amount is known about the molecular interactions that contribute to agonist binding, far less is known about the pathway between binding site and channel pore and the accompanying conformational changes that control gating and desensitization. Using unnatural amino acid mutagenesis, we have characterized the novel behavior of a subtle backbone mutation in loop D, an established contributor to the agonist binding site of the mouse muscle nAChR. When the two backbone mutations (α-hydroxytryptophan at γ55 and δ57) are combined with an M2 domain 9' Leu to Ser mutation (in the pore near the putative gate), macroscopic ACh dose-response relations show two components. Single-channel recordings show a similarly anomalous change in activation over a range of concentrations, as measured by P_{open}. Characterization of the contribution of this binding site mutation to the nAChR gating pathway may lead to a better understanding of the allosteric transitions that connect binding events to channel opening.

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1417-Pos Inter-loop Proline Serves as Hydrophobic Anchor in Coupling Agonist Binding to Channel Gating in Nicotinic Receptors

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Board B393

Nicotinic acetylcholine receptors (AChRs) mediate rapid excitatory synaptic transmission in the peripheral and central nervous systems. They convert binding of nerve-released ACh into opening of an ion channel intrinsic to the AChR, yet the structural basis for this triggering action is not fully understood. Our previous studies revealed a principal pathway linking binding to gating at the junction of binding and pore domains. It consists of an electrostatically coupled pair of residues Glu 45/Arg 209 linking the pre-M1 domain and the β1-β2 loop, and the hydrophobic residues Val 46, Pro 272 and Ser 269 linking these two domains to the pore via the M2-M3 loop. Here we investigate additional residues at the binding-pore interface using single channel kinetic analysis, site-directed mutagenesis and thermodynamic mutant cycle analysis. We find that in contributing to channel gating, Pro 272 in the M2-M3 loop is energetically coupled to Val 132 of the signature Cys-loop and Val 46 of the β1-β2 loop. Furthermore, we find that these three residues are optimized for efficient channel gating in both size and hydrophobicity, suggesting variations in these residues lead to diversity in gating efficiency among members of the Cys-loop receptor family. The overall results suggest Pro 272 serves as a hydrophobic anchor for the β1-β2 and Cys-loops in conveying ACh binding to the pore.

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1418-Pos Photoaffinity Labeling the α4β4 Neuronal Nicotinic Acetylcholine Receptor with the Agonist [^{125}I]Epibatidine

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Board B394

Neuronal nAChRs are key targets for potential therapeutic agents for neuropathic diseases, cognitive enhancement, analgesia, and nicotine dependence. The presence of either the β2 or the β4 subunit largely determines the unique pharmacological properties of heteromeric neuronal nAChRs. Nicotinic agonists including epibatidine have a higher affinity for the nAChR subtypes containing the β2 subunit compared with nAChR subtypes containing the β4 subunit. To directly study the molecular determinants that are responsible for these differences, membranes prepared from HEK 293 cells stably transfected with either human α4β2 or rat α4β4 nAChRs or affinity-purified and lipid reconstituted hα4β4 and rα4β2 receptors were photolabeled with the high affinity agonist [^{125}I]Epibatidine. [^{125}I]Epibatidine photoincorporated specifically into the β4 subunit with no (or insignificant) photoincorporation within α4 and β2 subunits. [^{125}I]Epibatidine photoincorporation in the β4 subunit was limited to the agonist binding pocket as the majority of labeling was displaceable by addition of either nicotine or epibatidine. Within the β4 subunit, the site of specific [^{125}I]Epibatidine photincorporation was mapped to two *S. aureus* V8 protease fragments of approximately 14 KDa (β4V8-14) and 6 KDa (β4V8-6). N-terminal sequencing of rpHPLC-purified β4V8-14 and β4V8-6 revealed that both fragments begin at β4Val^{102} and contain Loop E (β4Val^{110}-Pro^{120}) of the agonist binding domain. Based on the lack of [^{125}I]Epibatidine incorporation into the β2 subunit compared to the β4 subunit, a sequence comparison of the Loop E region of each subunit, and photoincorporation of [^{3}H]Azido-Epibatidine into Met^{116} within Loop E of the AChBP (1), we propose that β4Gln^{117} is the principal site of [^{125}I]Epibatidine labeling. Additional studies are currently in progress to further define the site(s) of [^{125}I]Epibatidine labeling in the α4β4 nAChR.

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1419-Pos Alcohol Can Remarkably Enhance Synaptic Impairment In The Slow Channel Congenital Myasthenic Syndrome

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Board B395

Slow-Channel Syndrome (SCS) is a neuromuscular disorder, characterized by a progressive muscular weakness due to a single amino acid substitution in muscle-type acetylcholine receptors (AChR). Our most recent data indicate that alcohol can enhance synaptic impairment in the SCS. Worsening of synaptic impairment by alcohol appears to be mutation-dependent. Effects of acute and chronic alcohol exposure were examined in three transgenic mice models for SCS mutations; εL269F, αL251T, αC418W and WT (FVB). We performed focal recordings of Miniature End-Plate currents (MEPC) on diaphragms of SCS mice during acute and chronic alcohol exposure. The endplate integrity was evaluated using a fluorescent alexa-488-α-Bungatoroxin and confocal imaging. The transgenic mice displayed a longer lasting MEPC decay time, as expected, from their delayed closing. In the presence of acute and chronic alcohol (0.10%) the MEPC decay time was remarkably increased for αC418W and εL269F. The MEPC decay time for WT and αL251T was not affected by acute or chronic alcohol exposure. Chronic alcohol exposure (10 days, 0.10%) produced a remarkable increase in endplate number and area of εL269F mice. For αL251T and WT mice we found no effect on the endplate integrity. According to present data, we hypothesize that alcohol consumption can contribute to different degrees of disease within the same SCS family. Summarizing, the present data suggests that alcohol may enhance the synaptic remodeling and disease for some SCS, and may lead to practical prevention strategies that will result in long-term benefit in SCS.

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1420-Pos Relationship Between Fret Efficiency And Oligomeric Size For M_2 Muscarinic Receptors In Live Cells

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Much evidence suggests that G protein-coupled receptors exist at least partly and perhaps wholly as oligomers. The latter often are referred to as dimers, but dimers often cannot be distinguished from larger oligomers. The number of subunits is of some interest, as it can be a determinant of properties, such as cooperativity, that have been implicated in the signaling process. In a new approach, photobleaching and acceptor-sensitized emission are used to determine the apparent efficiency of energy transfer between fluorophores within a population of oligomers in individual cells expressing different relative amounts of acceptor and donor ($[A]_t/[D]_t$). Those variables then are related in terms of a model that gives the true FRET efficiency (*E*) for a single donor-acceptor pair within an oligomer comprising *n* monomers. This approach was applied to M_2 receptors tagged at the *N*-terminus with the enhanced green, cyan, or yellow fluorescent protein (EGFP, ECFP or EYFP). The extracellular location of the fluorophore permits the control of environmental pH. The constructs were coexpressed in Chinese Hamster Ovary cells, and the fluorescence was monitored by laser-scanning confocal microscopy and spectral deconvolution. Apparent efficiency was determined by donor-dequenching and acceptor-sensitized emission; the corresponding value of $[A]_t/[D]_t$ was determined from empirically derived spectral properties. The data were analyzed in terms of the model to obtain estimates of *E* from essentially equivalent fits at different values of *n*. This approach therefore can be expected to reveal the size of the oligomer when used in conjunction with an independent estimate of *E*. Preliminary studies in which ECFP-M_2 was coexpressed with an EYFP-tagged, *N*-truncated variant of the Wnt receptor Frizzled-1 suggest that FRET from random collisions was negligible.

1421-Pos Spontaneous Conformational Changes And Toxin Binding In α7 Nicotinic Acetylcholine Receptor: Insight Into Channel Activation And Inhibition

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Nicotinic acetylcholine receptors (nAChRs) represent a paradigm for ligand-gated ion channels. Despite intensive studies over many years, our understanding of the mechanisms of activation and inhibition for nAChRs is still incomplete. Here we present molecular dynamics (MD) simulations of the α7 nAChR ligand-binding domain (LBD), both in apo form and in α-Cobratoxin bound form, starting from the respective homology models built on crystal structures of the acetylcholine binding protein. The toxin-bound form was relatively stable and its structure was validated by calculating mutational effects on the toxin binding affinity. On the other hand, one of the five apo subunits was seen to spontaneously move away from the starting conformation. This motion appears very similar to what has been proposed for leading to channel activation by agonists. At the top the C loop and the adjacent β7–β8 loop swing downward and inward while the bottom of the F loop, β9, and β10 swing in the opposite direction. These swings appear to tilt the whole subunit clockwise (side view). The resulting changes in solvent accessibility show strong correlation with experimental

results by the substituted cysteine accessibility method upon addition of acetylcholine. Our MD simulation results are consistent with a mechanistic model in which the apo form, while predominantly sampling the "closed" conformation, can make excursions into the "open" conformation. The open conformation has high affinity for agonists, leading to channel activation, whereas the closed conformation upon further distortion has high affinity for antagonists, leading to inhibition.

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1422-Pos Effects of Troponin Exchange on Length-Dependent Activation in Skinned Porcine Ventricular Muscle

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The basis of the Frank-Starling mechanism of the heart resides in the intrinsic ability of myocardial fibers to produce higher active force in response to stretch (i.e., length-dependent activation). We have reported that titin-based passive force operates as one of the triggering factors in this phenomenon. In the present study, we investigated whether or not length-dependent activation is modulated at the thin filament level. We used skinned porcine left ventricular muscle that had been treated with 1% (w/v) Triton X-100. Quasi-complete reconstitution of thin filaments with skeletal troponin (sTn; prepared from rabbit psoas muscle) increased Ca^{2+} sensitivity of force and attenuated length-dependent activation. A control experiment showed that treatment of skinned porcine ventricular muscle with exogenous cardiac troponin (prepared from porcine ventricular muscle) did not alter length-dependent activation. We then investigated the effect of sTn reconstitution on cross-bridge kinetics by measuring the rate of force redevelopment (k_{tr}). k_{tr} increased upon sTn reconstitution at submaximal levels, suggesting the acceleration of cross-bridge formation and, accordingly, a reduction in the fraction of resting cross-bridges that can potentially produce active force. An increase in titin-based passive force, induced by manipulating the pre-history of stretch, enhanced length-dependent activation, in both control and sTn-reconstituted muscle. These results suggest that troponin plays an important role in length-dependent activation via on-off switching of the thin filament state, in concert with titin-based regulation.

1423-Pos Coupled Interactions of Troponin C Ca^{2+}-Binding Kinetics and Strong Crossbridge Formation in Cardiac Muscle Contraction

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Striated muscle contractile regulation requires both Ca^{2+} binding to troponin C (TnC) and strong actomyosin crossbridge formation for thin filament activation and tension generation. We hypothesize that these processes are unique in skeletal and cardiac muscle and studied the coupled Ca^{2+} and crossbridge processes in demembranated rat cardiac trabeculae at 15°C. Ca^{2+} dissociation (k_{off}) from cardiac TnC (cTnC) was altered with whole cTn exchange using wild-type (WT) cTn (k_{off}=29.7±0.5s^{-1}, 15°C), L48Q cTn (7.3±0.1s^{-1}) or I61Q cTn (76.0±9.3s^{-1}). Strong crossbridge formation was reduced with 2,3-butandione monoxime (BDM) or inorganic phosphate (P_i) and increased with 2-deoxy-ATP (dATP). Maximal tension (F_{max}; pCa 4) after cTn exchange was ~85% for WT and L48Q cTn and ~20% for I61Q cTn. At saturating Ca^{2+} (pCa 4), 1–50mM BDM inhibited F_{max} with an inhibition constant (K_i) of 7.9±1.7mM for WT cTn, 9.3±1.5mM for L48Q cTn, and 5.0±1.4mM for I61Q cTn. F_{max} inhibition with 1–10mM P_i showed no difference in K_i between cTnC types (1.8±0.4mM for WT cTn), even though reconstituted F_{max} varied greatly. Increased crossbridge formation and cycling with dATP increased F_{max} by ~30% for WT and L48Q cTn and ~80% for I61Q cTn. In summary, when Ca^{2+} binding was increased (L48Q cTn), altering crossbridge formation or kinetics did not change the crossbridge component of thin filament activation. In contrast, reducing Ca^{2+} binding (I61Q cTn) resulted in greater capacity to increase activation via strong crossbridge formation but did not influence the ability to maintain activation as the crossbridge component was decreased. These data suggest that tight coupling exists between Ca^{2+} binding to cTn and strong crossbridge formation in cardiac muscle thin filament activation, unlike our previous results in skeletal muscle. Ongoing investigations include computational modeling approaches. HL61683, HL65497.

1424-Pos Measurements of Sarcomere Length in Intact Resting Rat Heart

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Cardiac contractility is modulated by sarcomere length (SL). While SL is a well-characterized parameter in fixed myocardium and isolated cells or tissue, little is known about SL-values in intact heart.

Two-photon fluorescence microscopy was used to measure SL up to 300μm sub-epicardially in rat whole hearts (n=12), excised after cervical dislocation and Langendorff-perfused within 150s. Hearts were placed in a tailor-made silicone cradle and gently stabilized by nylon mesh. Tissue was stained by coronary perfusion with di-4-ANEPPS (Invitrogen) in normal Tyrode (5μM over 5min), and cardioplegically arrested for imaging (room temperature) using a Leica TCS-MP2 multi-photon microscope (excitation 840nm, collection 400–700nm). Fluorescence intensity profiles were collected perpendicularly to user-defined paths along the axis of individual cells, and SL was analyzed by Fourier transform. Path alignment is