the same receptor mediates such opposing effects. To address this question, in this study we combined electrophysiological recordings of rat P2X7R current using an ultrafast application system for agonist delivery and removal and confocal imaging studies using the YFP-tagged P2X7R and CFP-tagged endoplasmic reticulum (ER) and Golgi markers. The rates of receptor activation and deactivation were consistent with a previously proposed hypothesis of high and low affinity ligand binding sites at P2X7Rs. Activation of high affinity sites resulted in low amplitude slowly desensitizing currents and internalization of receptors. On the other hand, activation of low affinity sites led to a secondary current growth and a sustained rise in calcium, the plasma membrane blebbing, and increase in the cell volume, resulting in cytolysis during the sustained receptor occupancy. These plasma membrane events were associated with broadening and separation of ER tubes, their fragmentation, vesiculation, and fusion of vesiculated ERs, whereas the structure of Golgi apparatus was not affected. Removal of agonist facilitated retraction of blebs and reversed the cytolytic cascade but did not stop the ongoing disruption of the ER morphology. These results suggest that the level of saturation of the ligand binding sites and duration of stimuli determines the nature of the P2X7R gating and actions. The results further indicate that the plasma membrane blebbing and cytolytic effects are independent of disruption of ER morphology and that the ER stress response is probably coupled with apoptosis.

3654-Pos

Redox Modulation of ATP-Gated P2X7 Currents

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Activation of P2X7 receptors is known to initiate downstream signaling processes including the release of proinflammatory cytokines and reactive oxygen species (ROS). Recombinant and native P2X7 receptors have been observed to exhibit time-dependent changes in current amplitude, an effect observed both during prolonged continuous ATP exposure and during short repeated applications of adenosine 5′-triphosphate (ATP). We used patch clamp electrophysiology in the whole cell perforated patch configuration to test the hypothesis that this time-dependent change in current amplitude reflected changes in the redox environment of membrane P2X7 receptors. In HEK293 cells expressing recombinant P2X7 receptors, we found that short repeated applications of ATP (1 s exposure every 60 s) evoked currents that increased and/or decreased in peak amplitude for several minutes before reaching a reproducible steady state amplitude. In cells that ultimately exhibited a net reduction in peak current amplitude over time, we observed that exposure to the membrane permeable reducing agent, DTT (1 mM, 1 min), significantly increased the peak current amplitude of subsequent ATP-evoked responses. We repeated the experiment with the endogenous reducing agent, glutathione, and this chemical also potentiated the amplitude of ATP-gated currents in these cells. In all cells, exposure to the oxidizing agent, hydrogen peroxide, was observed to reduce the amplitude of ATP-gated currents. In summary, ATP-gated currents through P2X7 receptors appear to be sensitive to modulation by redox chemicals.

3655-Pos

Identifying the Ion Access Pathway to the Transmembrane Pore in P2X Receptors

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P2X receptors are trimeric ion channels activated by extracellular ATP. Upon activation, P2X receptors promote inward current of cations to evoke action potentials or trigger calcium mediated signaling that are important for pain sensing, inflammation, and the synaptic transmission. However, the molecular mechanism of how extracellular ions access the transmembrane pore of P2X receptors is unknown. According to the zebrafish P2X4 crystal structure in the closed state, extracellular ions appear to be readily accessible to the pore through three identical fenestrations located right above the membrane leaflet (lateral pathway). In addition, ions may access the pore through a second possible pathway that runs through the central voids along the molecular three-fold axis of symmetry (central pathway). While this pathway is hypothetical, as the constrictions flanking the central voids are too narrow for hydrated ions to pass (~2.3 Å), agonist binding may expand these constrictions to enable ions to access the transmembrane pore. We have begun to explore the pathway ions use to move through the extracellular domain to enter the pore by inserting cysteine residues into rat P2X2 receptor channels and measuring reaction rates with a range of thiol-reactive reagents and ions. We found that MTSEA-Texas Red (MW=~750) can access T336 in the transmembrane pore with an apparently fast modification rate when the channel is open. These results suggest a large access pathway exists between the extracellular solution and the transmembrane pore, consistent with the fenestrations observed in the crystal structure. We are currently testing whether reagents of various size may also move through the central pathway.

3656-Pos

Characterization of Shark ASIC1b, an Ancient Form of an Acid-Sensing Ion Channel

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Acid-sensing ion channels (ASICs) are cation-permeable membrane proteins activated by extracellular H^+. They belong to the class of DEG/ENaC channels and share a common topology with cytosolic termini, two transmembrane domains and a large extracellular loop. ASICs are present in the genome of chordates but are absent in other animals. So far, functional ASICs that are gated by protons were cloned from bony fish, chicken and mammals. In contrast, ASICs from urochordates, jawless vertebrates, cartilaginous shark were shown to be H^+-insensitive, suggesting that proton-gating evolved relatively late in bony fish and that primitive ASICs have a different gating mechanism. Recently, amino acids that are crucial for proton-gating of ratASIC1a have been identified; these amino acids are conserved in an ASIC from the shark *Squalus acanthias* (sharkASIC1b).

Here we show that, contrary to previous findings, sharkASIC1b is gated by protons. This result shows that the conservation of the amino acids crucial for proton-gating can predict proton-sensitivity of an ASIC. The sharkASIC1b current is half-maximally activated pH 6.0 and is blocked by amiloride. It desensitizes quickly but incompletely, efficiently encoding transient as well as sustained proton signals at pH values between 7.0 and 6.2.

Since ratASIC1a desensitizes approximately 100-fold slower than sharkASIC1b but completely, we started a chimeric approach swapping regions between sharkASIC1b and ratASIC1a to identify the amino acids determining speed of desensitization and the unique sustained current. Functional chimeric channels point towards two separate regions in the large extracellular domain accounting for these two characteristics.

3657-Pos

The β1-β2 Linker in the Extracellular Domain of ASIC1 Determines Desensitization of ASIC1

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ASICs are proton-activated channels expressed in the nervous system of all chordates. Despite high amino acid conservation of the ASICs we have observed significant functional differences among these channels. For instance, ASIC1 from fishes and amphibian are more sensitive to desensitization by preconditioning pH than the mammalian channels. Studies of fish, shark, and frog ASIC1 show that these channels are completely desensitized by pH $\geq$7.3. We identified three residues in the linker connecting b1 to b2 in the extracellular domain of ASIC1 (corresponding to positions P82, N83, and M84 in the frog sequence) that are responsible to this property. Mutations of those residues for the corresponding ones in rat ASIC1 shift the preconditioning pH from 7.5 to 7.3 and decrease the rate of decay of the peak currents from 3.5 ± 0.2 s-1 to 1.6 ± 0.2-1. Out of the three indicated residues, the one in position 84 has the largest effect in desensitization. Similar results were obtained in shark ASIC1 and in elephant shark ASIC1. We conclude that the b1-b2 linker is an important determinant of the rate of ASIC1 desensitization thereby it may undergo conformational changes during the desensitization process. From a physiological point of view, the b1-b2 linker sets the pH range wherein these channels are functional. The results are also consistent with the notion that the b1-b2 linker has evolved to optimize the response of ASIC1 to the range of physiological extracellular pH of each species, which is higher in amphibians and fishes than in mammals.

3658-Pos

Multisite Binding of Anesthetics to GLIC, a Pentameric Ligand-Gated Ion Channel

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Volatile and intravenous anesthetics inhibit channel function of nicotinic acetylcholine receptors (nAChRs).Here we report the putative general anesthetic binding sites in *Gloeobacter vioaceus* pentameric ligand-gated ion channel (GLIC), a bacterial homolog of nAChR, using fluorescence quenching, multi-ns molecular dynamics (MD), and docking analysis. Fluorescence

quenching results of halothane (Q_{max}~ 85%, K_d ~ 2 mM^{-1}) and thiopental (Q_{max}~ 100%, K_d ~ 0.1 mM^{-1}) suggest these two anesthetics bind to almost all TRPs. However, ketamine and etomidate only quenched about 20 ~ 30% with nonspecific binding, indicating other non-TRP binding sites may exist. X-ray structure of GLIC (PDB: 3EAM) reveals three tryptophan (TRP) sites: A) W47 and W72 in the extracellular domain (ECD); B) W160 at the interface between ECD and transmembrane domain (TMD); and C) W213 and W217 in the TMD. Fluorescence quenching of three site-directed mutants, where A, B, or C site was singly remained, demonstrated similar binding affinities to all three sites for both halothane (Q_{max}: 84 ~ 90%, K_d : 1.3 ~ 2.1 mM^{-1}) and thiopental (Q_{max}: 94 ~ 100%, K_d : 0.07 ~ 0.1 mM^{-1}). Of these three sites, halothane prefers W47 and W72 ($Q_{max} = 90 \pm 2\%$, $K_d = 1.3 \pm 0.2$ mM^{-1}) while thiopental prefers W160 ($Q_{max} = 94 \pm 2\%$, $K_d = 0.067 \pm 0.005$ mM^{-1}). In consistent with fluorescence quenching experiments, our theoretical study also finds that both anesthetics bind to all TRPs though both prefer W160. Furthermore, non-TRP sites are discovered by our theoretical study: one near the TM2-3 loop and the other near D86 in the ECD. Collectively, This investigation will elucidate where volatile and intravenous anesthetics interact with pLGICs and reveal how the binding may lead channel functional changes. (Supported by NIH R01GM056257, R01GM069766, R37GM049202, R01GM066358).

3659-Pos

Mutational Analysis of the Prokaryotic Pentameric Ligand-Gated Ion Channel GLIC

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For pentameric ligand-gated ion channels (pLGICs), the movements involved in coupling neurotransmitter binding to channel activation are unclear. The crystal structures of the prokaryotic pLGICs homologs from *E. chrysanthemi* (ELIC) and *G. violaceus* (GLIC) in presumed closed and open channel conformations provide unparalleled opportunities to explore how ligand binding triggers channel opening. GLIC is a proton-gated channel. To test the hypothesis that the proton binding site in GLIC is located in an analogous position as the agonist binding sites of eukaryotic pLGICs and is formed, in part, by acidic amino acids, we neutralized acidic residues in Loops A (E74Q), C (E176Q, D177N, E180Q), and F (D144N, E146Q). Two-electrode voltage clamp recordings from wild-type and mutant GLIC channels expressed in *Xenopus* oocytes showed that the mutations had no effect on proton-mediated channel gating (pH_{50}, pH_{max}) suggesting that titration of these carboxylates is not involved in the proton-dependent gating of GLIC and that the proton binding site is located elsewhere.

We are using fluorescence recording of site-specific labels in GLIC expressed in *Xenopus* oocytes combined with two-electrode voltage clamping to monitor the motions in GLIC associated with channel opening.We made cysteine mutations in a cys-less GLIC (C26A) background in loop 2 (K32C), loop 9 (T157C), M2 helix (E242C, T243C, N244C) and M2-M3 loop (K247C, P249C). The cysteine mutant receptors were labeled with the sulfhydryl-reactive, environmentally-sensitive fluorescent probe Alexafluor 546 C_5-maleimide. Combined voltage clamp and fluorometry monitor proton-induced channel activity and local protein movements simultaneously. Application of pH 5 buffer decreased fluorescence at T157C, K247C and P249C suggesting that these residues become more exposed to a hydrophilic or aqueous environment consistent with a movement of the M2-M3 loop upwards and outward on channel activation.

3660-Pos

Molecular Dynamics Investigation of Anesthetic Halothane Interactions with the Proton-Gated Ion Channel GLIC

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Experimental studies found that general anesthetics inhibited the function of GLIC. To investigate the underlying inhibition mechanism, we studied the interaction of halothane with GLIC and determined the potential of mean force (PMF) for transporting a sodium ion across the GLIC channel in the presence and absence of halothane. Multiple halothane binding sites were identified through docking and multi-nanosecond molecular dynamics (MD) simulations; three important ones are: A) inter-subunit site near TM2-3 loop; B) inter-subunit site near D86; and C) intra-subunit site near W160. MD simulations demonstrated that halothane altered GLIC dynamics and electrostatic interactions between critical residues. Halothane near W160 reduced the stability of salt-bridges between D32 and R192, whose homologous electrostatic interaction was suggested to be important for channel gating in the ligand-gated ion channels. To assess potential impact of halothane binding to channel conductance, we calculated PMF using adaptive biased force method. Protonation state of E222 is a determinant for the PMF. PMF profile with five deprotonated E222 residues in the pentamer showed a significantly deeper energy well than that with three deprotonated E222, suggesting that five deprotonated E222 may trap an ion and that partial protonation of E222 is necessary for ion leaving the trap. While halothane near sites A and B introduced comparatively small effects on PMF, a profound PMF change near E222 was observed in the presence of halothane at W160. This change may likely affect the single channel conductance. Taken together, halothane may modulate GLIC function through altering salt bridges crucial for gating, coupled with a breaking of the fivefold symmetry of the pore-lining helices, leading to a change in the energy profile for ion passage through the channel. Supported by NIH (R01GM66358 and R01GM56257) and NCSA through PSC.

3661-Pos

Packing of the Extracellular Domain Hydrophobic Core is Evolutionarily Optimized to Facilitate Pentameric Ligand-Gated Ion Channel Activation

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Protein function depends on protein dynamics as well as structure. Local loose packing of hydrophobic cores is not infrequent in proteins, as the enhanced flexibility likely contributes to their biological function. The crystal structure of the extracellular domain of the nicotinic acetylcholine receptor (nAChR) α1 subunit revealed a hydrophilic water-filled cavity formed by Thr-52 and Ser-126 buried in the hydrophobic core of the protein. Mutation of these residues to bulky hydrophobic amino acids substantially reduced acetylcholine activated channel current suggesting loose-packing of the β-sandwich core is important for nAChR function. Intriguingly, structure-based sequence alignment suggests the presence of loose packing of the hydrophobic β-sandwich core in other pentameric ligand-gated ion channels (pLGICs), whereas tight packing is observed in the crystal structures of the nonchannel homolog, acetylcholine binding protein (AChBP). Here, we examined the generality and importance of this loose packing for pLGIC function using experimental and computational approaches. Mutating aligned residues in the related heteropentameric GABA-A receptor disrupted GABA-mediated currents. Mutations in the β2 subunit had the largest effects suggesting distinct requirements for subunit flexibility in receptor activation. Using FoldX, we examined the energetic cost of mutating residues in the hydrophobic core on protein folding in AChBP, prokaryotic and eukaryotic pLGICs as a measure of protein stability. Interestingly, a loss in protein stability appears correlated to the ability of pLGICs to rapidly switch from closed to open channel states in the presence of ligand. Overall, we suggest that loose packing of the hydrophobic core likely developed as an evolutionary strategy aimed to optimize the specialized allosteric mechanisms of pLGICs.

3662-Pos

Site-Specific Fluorescence Reveals Distinct Structural Changes Induced in the Human ρ1 GABA Receptor by Inhibitory Neurosteroids

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The ρ1 GABA receptor is inhibited by a number of neuroactive steroids. A previous study (Li et al., 2007, JPET 323:236-247) focusing on the electrophysiological effects of inhibitory steroids on the ρ1 receptor found that steroid inhibitors could be divided into three major groups based on how mutations to residues in the M2 transmembrane domain modified inhibition. It was proposed that the steroids act through distinct mechanisms. We have selected representatives of the three groups (β-estradiol, pregnanolone and pregnanolone sulfate), and probed how these steroids modify fluorescence changes from the Alexa 546 C5 maleimide fluorophore attached to residues in the extracellular region of the receptor. The results indicate that the steroids have distinct effects on fluorescence changes. Pregnanolone sulfate diminished the fluorescence change produced by GABA at the K217C and S66C positions. The application of β-estradiol reduced the fluorescence change at the L166C and S66C positions. Introduction of the T298F mutation abolished the β-estradiol-induced reduction in fluorescence change at the L166C and S66C sites, and also abolished the functional inhibition produced by the steroid. Pregnanolone did not affect fluorescence changes at any of the sites examined. The findings are consistent with the steroids acting as allosteric inhibitors of the ρ1 GABA receptor, and support the hypothesis that divergent mechanisms underlie the action of inhibitory steroids on the ρ1 GABA receptor.