results suggest that the divergent selectivities observed for endogenous store-operated channels might involve a heteromeric Orai1-Orai3 channel complex. Supported by FWF P18169.

2880-Plat

Fast Ca^{2+} Dependent Inactivation Of CRAC Channels Requires A Cytosolic Region Of STIM1

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The distinguishing biophysical features of mammalian Ca^{2+}-release activated Ca^{2+} (CRAC) channels include high Ca^{2+} selectivity, small unitary conductance, and fast Ca^{2+}-dependent inactivation on a millisecond time scale. Our previous studies of fast inactivation in Jurkat T cells suggested that Ca^{2+} binds to sites several nanometers from the intracellular mouth of the CRAC channel pore, possibly on the channel itself. The identification of STIM1 as the ER Ca^{2+} sensor and Orai1 as the pore-forming subunit of the CRAC channel has enabled studies of the molecular basis of activation and inactivation. We have recently identified a 107-residue cytosolic STIM1 fragment corresponding to the minimal STIM1 domain required for activation of the CRAC channel. The CRAC activation domain, or CAD, binds directly to Orai1 to activate CRAC current to the same mean level as wild-type STIM1, but while bypassing store depletion. CRAC currents were measured by whole-cell patch-clamp electrophysiology in HEK 293 cells coexpressing human Orai1 with a range of constructs derived from the cytosolic region of human STIM1. CAD-induced CRAC currents retain high Ca^{2+} selectivity, but surprisingly lack fast Ca^{2+}-dependent inactivation, revealing a critical role for STIM1 in the inactivation gating process. Truncating STIM1 at the C-terminal end of CAD also yielded currents without fast inactivation in store-depleted cells. Extending CAD in the C-terminal direction partially reconstituted fast inactivation, but full reconstitution required both C- and N-terminal extensions of CAD. We conclude that a domain of STIM1 C-terminal to CAD is absolutely required for fast Ca^{2+}-dependent inactivation of the CRAC channel. Elements of STIM1 N-terminal to CAD may enhance fast inactivation, possibly by increasing the local density of CRAC channels and Ca^{2+} influx, or by concentrating critical STIM1 domains near the inner pore mouth.

2881-Plat

Differential Modulation of Type-1, Type-2 and Type-3 Inositol (1,4,5)-Trisphosphate Receptors by ATP

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Inositol (1,4,5)-trisphosphate receptors ($InsP_3R$) are the predominant route of Ca^{2+} release in non-excitable cells and they play a vital role in regulating intracellular Ca^{2+} signals. There are three isoforms ($InsP_3R$-1, $InsP_3R$-2 and $InsP_3R$-3) of $InsP_3R$ expressed in mammalian cells. This sequence diversity along with varied tissue distributions suggests that there are isoform-specific regulatory mechanisms. One such regulatory mechanism is the modulation of Ca^{2+} release from $InsP_3R$ by cytosolic ATP. ATP positively regulates all three $InsP_3R$ isoforms, but with distinct functional characteristics. We found that ATP was required for maximal $InsP_3$-induced Ca^{2+} release from $InsP_3R$-1 and $InsP_3R$-3 while $InsP_3R$-2 attained maximal activity in the absence of ATP. Furthermore, $InsP_3R$-2 was more sensitive to ATP modulation than either $InsP_3R$-1 or $InsP_3R$-3. All three isoforms contain putative ATP binding domains, but the contributions of these sites to ATP modulation of $InsP_3R$ are poorly understood. $InsP_3R$-1 contains two predicted ATP binding domains (ATPA, and ATPB) while $InsP_3R$-2 and $InsP_3R$-3 each express a single ATPB site. We examined the contributions of these ATP binding sites to the subtype-specific effects of ATP on $InsP_3R$ isoforms. ER Ca^{2+} measurements from permeabilized DT40 cells and single channel recordings of $InsP_3R$ were used to measure the effects of ATP on wild-type and mutated $InsP_3R$. We found that ablation of the ATPB site in $InsP_3R$-2 eliminated the enhancing effects of ATP on this isoform. Surprisingly, the positive effects of ATP were retained in $InsP_3R$-1 and $InsP_3R$-3 devoid of their respective ATP binding sites. ATP, therefore, differentially regulates the three $InsP_3R$ isoforms and likely regulates $InsP_3R$-1 and $InsP_3R$-3 via novel ATP binding sites. The implications of this differential regulation on Ca^{2+} signals would likely be determined by the relative ratios of the three isoforms expressed in a given cell.

2882-Plat

Functional Stoichiometry Of The Unitary Calcium-release-activated Calcium Channel Revealed By Single-molecule Imaging

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Two proteins, STIM1 in the endoplasmic reticulum and Orai1 in the plasma membrane, are required for the activation of Ca^{2+} releaseactivated Ca^{2+} (CRAC) channels at the cell surface. How these proteins interact to assemble functional CRAC channels has remained uncertain. Here, we determine how many Orai1 and STIM1 molecules are required to form a functional CRAC channel.

We engineered several genetically expressed fluorescent Orai1 tandem multimers and a fluorescent, constitutively active STIM1 mutant. The tandem multimers assembled into CRAC channels, as seen by rectifying inward currents and by cytoplasmic calcium elevations. CRAC channels were visualized as fluorescent puncta in total internal reflection microscopy. With single-molecule imaging techniques, it was possible to observe photo-bleaching of individual fluorophores and to count the steps of bleaching as a measure of the stoichiometry of each CRAC channel complex. We conclude that the subunit stoichiometry in an active CRAC channel is four Orai1 molecules and two STIM1 molecules. Fluorescence resonance energy transfer experiments also showed that four Orai1 subunits form the assembled channel. From the fluorescence intensity of single fluorophores, we could estimate that our transfected HEK293 cells had almost 400,000 CRAC channels and that, when intracellular Ca^{2+} stores were depleted, the channels clustered in aggregates containing ~1,300 channels, amplifying the local Ca^{2+} entry.

Platform BB: Channel Regulation & Modulation

2883-Plat

Disruption Of Interactions Between AKAP79/150 And KCNQ K^+ Channels By Ca^{2+}/Calmodulin Observed Using TIRF/FRET

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KCNQ2-5 subunits encode the M-current, a K^+ conductance that controls neuronal excitability. Stimulation of $G_{q/11}$-coupled receptors depresses M current via multiple intracellular signals, including depletion of PIP_2, generation of Ca^{2+}/calmodulin, and phosphorylation of KCNQ2 by PKC, recruited to the channels by AKAP79/150. We examined the interplay between these signals via FRET measurements performed under total internal reflection fluorescence (TIRF) microscopy, in which mostly membrane events are isolated. CHO cells were transfected with CFP-tagged KCNQ2-4, and a YFP-tagged construct containing the first 153 residues of AKAP79 ($AKAP79_{1-153}$) shown to be sufficient for binding to KCNQ2, PKC and receptors (Hoshi et al., *Nat Cell Biol*. **7**:1066-73). We found significant FRET between all KCNQ2-4 subunits and $AKAP79_{1-153}$ (13 $\pm$ 1.3%, 7.1 $\pm$ 2.0% and 10.1 $\pm$ 2.0% for KCNQ2-4, respectively). Since Ca^{2+}/calmodulin binding not only inhibits M channels (Gamper and Shapiro, *JGP* 122:17-31), but also acts on AKAP79/150 (Faux and Scott, *JBC* 272:17038-17044), we asked whether calmodulin alters KCNQ channel-AKAP79/150 interactions. Indeed, FRET between all CFP-KCNQ2-4 subunits and YFP-$AKAP79_{1-153}$ was much less in cells also co-transfected with wild-type calmodulin, but not when dominant-negative calmodulin was used that cannot bind Ca^{2+}. FRET was also substantial between the CFP-KCNQ2 (R345E) mutant that cannot bind calmodulin and YFP-$AKAP79_{1-153}$, which was however not reduced by calmodulin co-expression. Furthermore, the FRET between KCNQ2-4 and $AKAP79_{1-153}$ was also not reduced when the cells were depleted of PIP_2 by co-expression of a PIP_2 phosphatase. We conclude that calcified, but not apo, calmodulin interferes with KCNQ subunit-AKP79/150 interactions by binding to the channels, likely reducing their affinity for AKAP79/150, and that M channel-AKAP79/150 interactions may serve to anchor PKC to M-channel containing microdomains, where it stands poised to phosphorylate the channels upon stimulation of appropriate $G_{q/11}$-coupled receptors.

2884-Plat

Are Voltage-gated Potassium (Kv) Channels Recruited Into Lipid Rafts In Mammalian Brain Neurons?

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Differential sub-cellular distribution and voltage-dependent gating properties of Kv channels are crucial for the regulation of neuronal excitability. In mammalian central neurons, the majority of delayed-rectifier K+ currents (IK) are contributed by Kv2.1 channels, and Kv4.2 and Kv4.3 channels constitute most of the A-type K+ currents (IA) in the soma and dendrites. Kv2.1 channels are localized in distinct cell surface clusters in the soma and proximal dendrites,

a pattern conserved in recombinant channels expressed in HEK293 cells. This clustering is dependent on the extent of channel phosphorylation. Our recent studies have shown that the clustered localization and gating properties of Kv2.1 are dynamically regulated by altered neuronal activity, ischemia, and by neuromodulatory stimuli via Ca2+/calcineurin-mediated dephosphorylation of the channel protein. These changes in Kv2.1 play a neuroprotective role. A number of studies have proposed the association of Kv2.1 with caveolar lipid rafts. We observed that Kv2.1 clusters in HEK293 cells do not overlap with caveolin1- or flotillin1/2-containing lipid rafts/microdomains on the cell surface. Moreover, caveolin1 staining/puncta were detected only in cultured rat astrocytes/glia, not in cultured rat hippocampal neurons. Caveolin1-RFP, overexpressed both in HEK293 cells stably expressing Kv2.1, and in cultured hippocampal neurons, exhibited distinct surface puncta that did not overlap with Kv2.1 clusters, and also did not alter the current density and voltage-dependent channel gating properties. Cyclodextrin-induced disruption of lipid rafts did not alter the clustered localization of Kv2.1 in HEK293 cells. Similarly, the staining pattern of Kv4.2 and Kv4.3 channels with or without the overexpression of KChIP2 did not overlap with caveolin1- and flotillin1/2-containing lipid rafts/microdomains in HEK293 and COS cells. These results suggest that in mammalian central neurons, somatodendritic Kv channels are not recruited to, and function independently of, lipid rafts/microdomains.

2885-Plat

Stim-regulated Assembly And Stoichiometry Of The CRAC Channel Subunit Orai

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Recent RNAi screens have identified Stim and Orai as critical components of the Ca^{2+} release-activated Ca^{2+} (CRAC) channel. Stim senses depletion of the Endoplasmic Reticulum (ER) Ca^{2+} store, translocates from the ER to junctions adjacent to the plasma membrane (PM), and activates Orai pore-forming channel subunits in the PM to open the CRAC channel. The Orai oligomerization interface was investigated by co-immunoprecipitation of N-and/or C-terminal Orai deletion mutants and expressed Orai N- and C-terminal fragments. The transmembrane core domain plays a predominant role in subunit assembly; a weaker interaction interface was identified at the N-terminal region. We analyzed the quaternary structure of the Orai subunit and showed by cross-linking, and by non-denaturing gel electrophoresis that Orai is predominantly a dimer under resting conditions with or without co-expression of Stim. Single-molecule imaging of GFP-tagged Orai expressed in *Xenopus* oocytes revealed predominantly two-step photo-bleaching, consistent with a dimeric basal state. In contrast, co-expression of GFP-tagged Orai with the C-terminus of Stim as a cytosolic protein to activate the Orai channel without inducing Ca^{2+} store depletion or clustering of Orai into punctae yielded predominantly four-step photobleaching, consistent with a tetrameric Orai stoichiometry of the active CRAC channel. Interaction of the Orai C-terminal coiled-coil domain (as shown by structure-disruptive mutations) with the C-terminus of Stim thus induces Orai dimers to dimerize, forming a tetramer that constitutes the Ca^{2+}-selective pore. This represents a novel mechanism in which assembly and activation of the functional ion channel are mediated by the same triggering molecule and may reveal a new channel gating mechanism. New data will be presented on the Stim-Orai stoichiometry and activation mechanism.

2886-Plat

Protein Histidine Phosphatase 1 Negatively Regulates CD4 T Cells by Inhibiting the K+ Channel KCa3.1

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The calcium activated K+ channel KCa3.1 plays an important role in T lymphocyte Ca2+ signaling by helping to maintain a negative membrane potential which provides an electrochemical gradient to drive Ca2+ influx. We previously showed that Nucleoside Diphosphate Kinase Beta (NDPK-B), a mammalian histidine kinase, is required for KCa3.1 channel activation in human CD4 T lymphocytes. We now show that the mammalian protein histidine phosphatase (PHP)-1 directly binds and inhibits KCa3.1 by dephosphorylating histidine 358 on KCa3.1. Overexpression of wild type, but not a phosphatase dead PHPT-1 inhibited KCa3.1 channel activity. Decreased expression of PHPT-1 by siRNA in human CD4 T cells resulted in an increase in KCa3.1 channel activity and increased Ca2+ influx and proliferation following T cell receptor (TCR) activation indicating that endogenous PHPT-1 functions to negatively regulate CD4 T cells. Our findings provide the first example of a mammalian histidine phosphatase negatively regulating TCR signaling and are one of the few examples of histidine phosphorylation/dephosphorylation influencing a biological process in mammals.

2887-Plat

Targeting The Voltage Sensor of Kv7 channels: Novel Strategies to Cure Hyperexcitability Disorders

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The pore and gate regions of ion channels have been often targeted with drugs acting as channel blockers or openers. In contrast, the voltage sensing domain (VSD) was practically not exploited for therapeutic purposes. We recently designed a series of novel diphenylamine carboxylate derivatives to generate powerful Kv7.2 channel openers and blockers. Openers like the compound NH29 with aromatic nitro groups, robustly increased Kv7.2 K^+ currents. In sensory DRG and hippocampal neurons, the opener depressed evoked spike discharges. NH29 dampened hippocampal glutamate and GABA release thereby inhibiting spontaneous EPSCs and IPSCs. *In vivo*, these openers exhibited anti-convulsant activity. To identify the target residues involved in the action of NH29 we designed various mutations of Kv7.2 channels and checked the potency of NH29 using the whole-cell patch-clamp technique in CHO cells. We found that the mutants S121A at S1-S2 loop and L197G, R198A, R201A, R207W and R214W at S4 helix are significantly less sensitive to the activating effect of NH29 compared to WT Kv7.2 channels. Interestingly, our results indicate that NH29 does not act at the same target site as the opener retigabine (W236) and zinc-pyrithione. Docking experiments suggest that the nitro functionality of NH29 acts as a H-bonding acceptor which interacts with the guanidinium group of arginine 207 of the S4 helix. Thus, the new Kv7.2 channel opener NH29 acts as a gating modifier which interacts with the externally accessible surface of the VSD. NH29 docks to the groove formed by the interface between S4 helix and S1-S2, thereby stabilizing the VSD in the activated conformation. Our research is expected to generate a new generation of gating-modifiers specifically targeted to the VSD of potassium channels for the treatment of hyperexcitability disorders.

2888-Plat

Regulation of the NALCN Sodium Leak Channel by Neuropeptides

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Several neurotransmitters act through G-protein coupled receptors (GPCR) to evoke a ''slow'' excitation of neurons. These include peptides, such as substance P (SP) and neurotensin (NT), as well as acetylcholine and noradrenaline. Unlike the fast (~ ms) ionotropic actions of small molecule neurotransmitters, the slow excitation is poorly understood at the molecular level, but can be mainly attributed to suppressing K^+ currents and/or activating a non-selective cation channel. Several K^+ channels, including members in the K_V7 subfamily, are inhibited by the neurotransmitters in a G protein-dependent fashion. The molecular identity of the cation channel has yet to be determined; similarly how the channel is activated and its relative contribution to neuronal excitability induced by the neuropeptides are unknown. We show that, in the hippocampal and ventral tegmental area neurons, SP and NT activate the voltage-independent Na^+-leak channel NALCN, a unique member in the 4×6TM channel family that also includes the voltage-gated Ca^{2+} (Ca_V) and Na^+ (Na_V) channels. The activation by SP through NK1R (a GPCR receptor for SP) is not blocked by the non-hydrolyzable GTP or GDP analogs. These findings identify NALCN as the cation channel activated by SP receptor, and suggest that a G protein-independent mechanism is involved in the coupling from receptor to channel.

2889-Plat

Role of Aromatic Residues for Local Anaesthetic Binding to Ion Channels

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Local anesthetic and antiepileptic drugs acting on Nav and hERG channels have been assumed to bind to aromatic residues in the internal vestibule; to 1764F and 1771Y in Nav (Liu et al., 1996) and to 652Y and 656F in hERG (Mitcheson et al., 2000). Despite a lack of such residues in Kv channels, some local anaesthetics (e.g. bupivacaine) bind to Kv channels with a considerable affinity. To explore the role of aromatic residues for local anaesthetic binding we investigated the effect of bupivacaine on mutated Shaker channels (P466F and V473Y; I470Y and P474F), expressed in Xenopus oocytes, with a voltage clamp technique.