product only appears when the glass is dry. The results are consistent with water being an effective competitor for the ferric heme site in the presence of nitrate.

Platform BA: Calcium Signaling Pathways

2875-Plat

Isoform-specific Regulation Of The Ca-sensitive Transcription Factor NFAT In The Cardiovascular System

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NFAT transcription factors (Nuclear Factor of Activated T-cells) mediate Ca-sensitive gene transcription and are involved in cardiovascular remodelling. Nuclear localization of NFAT is dynamically regulated by intracellular Ca signals yielding to dephosphorylation and nuclear translocation of NFAT, and activity of intracellular kinases that induce nuclear export. The aim of this study was to analyze the regulation of NFAT in vascular endothelial cells and adult cardiomyocytes. Subcellular distribution of NFAT-GFP fusion proteins (isoforms NFATc1 and NFATc3) was analyzed with confocal microscopy and intracellular Ca ($[Ca]_i$) was measured simultaneously using rhod-2. In calf pulmonary arterial endothelial (CPAE) cells, application of ATP (5 μM) induced nuclear localization of both isoforms (quantified as an increase in $NFAT_{NUC}/NFAT_{CYT}$ ratio). Subsequent attenuation of $[Ca]_i$ to facilitate nuclear export resulted in substantial export of NFATc3 to the cytoplasm, which was sensitive to Leptomycin B (40 nM). Previously translocated NFATc1 was only partially affected by nuclear export, indicating isoform-specific regulation of NFAT in endothelial cells.

In cardiac myocytes regulation of NFAT was isoform-, and tissue-specific: NFATc1 displayed nuclear localization in quiescent myocytes, which was dependent on $[Ca]_i$ and further enhanced by blocking nuclear export (Leptomycin B) or by inhibition of intracellular kinases (20 mM LiCl, 1 μM alsterpaullone or 1 μM SP600125). In contrast, NFATc3 was distributed in the cytoplasm of quiescent cells. Incubation with Leptomycin B, but not inhibition of nuclear kinases induced nuclear localization of NFATc3 in ventricular cells. Incubation with the G_q protein-coupled receptor agonists endothelin-1 (100 nM) and Ang II (2 μM) induced nuclear localization of NFATc3 only in atrial, but not ventricular cells. We conclude that (i) regulation of nuclear NFAT in the cardiovascular system is isoform- and tissue specific and (ii) dynamically regulated by activity of nuclear export pathways.

2876-Plat

Alterations In Binding Properties Of Myocardial Nuclear Membrane Receptors Induce Nuclear Calcium Overload In Rat Ischemia-reperfusion

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Cell nuclei possess an independent calcium regulatory system consisting of nuclear Ca^{2+}-ATPases (NCA), IP_3 receptors (IP_3R), IP_4 receptors (IP_4R), ryanodine receptors(RyR), and nuclear pore complexes(NPC). We studied the changes in $Ca^{2+}{}_n$ and its regulatory system in rat model of myocardial ischemia-reperfusion injury(IRI) induced by 30 min coronary occlusion followed by 180 min reperfusion. The $Ca^{2+}{}_n$ content in isolated nuclei was measured with atomic absorption spectrophotometer. NPC permeability was assessed through the amount of calmodulin conjugated Alexa FluoTM 488 as fluorescent probes. NCA activity was evaluated by phosphate group released from ATP in enzymatic reaction. The maximum binding capacity(B_{max}) and dissociation constant(K_d) of IP_3R, IP_4R and RyR were determined by radioligand binding of [^{3}H]IP_4, [^{3}H]IP_4 and [^{3}H]-ryanodine to isolated cardiomyocytic nuclei. All results are shown in the table. Our findings suggest that in vivo rat myocardial IRI is characterized by $Ca^{2+}{}_n$ overload, upregulations of nuclear IP_3R and IP_4R, downregulations of NCA activity and RyR, and an increase in permeability of NPC. The upregulation of nuclear IP_3R and IP_4R may be responsible for the nuclear calcium overload.

2877-Plat

ATP-evoked Ca2 ± waves Stimulate Gene Expression In Human Airway Fibroblasts

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We sought to investigate the effects of a variety of autacoids on Ca^{2+}-handling in human airway fibroblasts. Primary cultured fibroblasts were loaded with the Ca^{2+}-indicator dye fluo-4 and studied using confocal fluorimetric microscopy. ATP (10^{-5} M) evoked recurring Ca^{2+}-waves . This fluorimetric change was greater and longer lasting within the nucleus of the cell than in the non-nuclear portion of the cytosol, and was only sometimes accompanied by a contraction. These responses were completely occluded by cyclopiazonic acid (10^{-5} M; depletes the internal Ca^{2+}-store) or the phospholipase C inhibitor U73122 (10^{-6} M). Pretreatment of the cells with ryanodine (10^{-5} M), on the other hand, had no effect on the ATP-evoked responses. With respect to the receptor through which this response was exerted, we found it to be mimicked by UTP or ADP but not by adenosine or α,β-methylene-ATP, and to be blocked by the purinergic receptor blocker PPADS; interestingly, PPADS itself appeared to sometimes evoke a rise in $[Ca^{2+}]_i$ on its own. ATP also evoked a membrane conductance change with characteristics of a non-selective cation current, markedly enhanced synthesis of the cytokine TGFβ and the matrix proteins fibronectin and collagen I; these changes in protein synthesis were blocked by PPADs and were partially reduced by ryanodine. We conclude that, in human pulmonary fibroblasts, ATP acts upon P2Y receptors to liberate internal Ca^{2+} through ryanodine-insensitive channels, leading to a Ca^{2+}-wave which courses throughout the cell and triggers protein synthesis.

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2878-Plat

Store-operated Ca^{2+} Entry Is Suppressed During Mitosis Due To Phosphorylation Of The Endoplasmic Reticulum Ca^{2+} Sensor Stim1

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When ER Ca^{2+} stores are depleted due to physiological Ca^{2+} release or pharmacological perturbation, Ca^{2+} influx via plasma membrane Ca^{2+} channels is activated by a process known as store-operated Ca^{2+} entry (SOCE). The current associated with SOCE is Ca^{2+} release-activated Ca^{2+} current (I_{crac}). SOCE involves Orai Ca^{2+} influx channels and STIM ER Ca^{2+} sensors. When ER Ca^{2+} stores are full, STIM1 is localized throughout the ER membrane; however, ER Ca^{2+} store depletion induces rearrangement of STIM1 into punctate structures near the plasma membrane where it activates Orai channels. Interestingly, mitosis is the only known physiological situation in which Ca^{2+} store depletion is dissociated from SOCE or I_{crac} activation. Identification of the molecular components of the SOCE signaling pathway has facilitated analysis of the mechanism underlying mitotic SOCE suppression. We found that in mitotic HeLa cells, an enhanced yellow fluorescent protein-tagged STIM1 (eYFP-STIM1) did not rearrange into puncta in response to Ca^{2+} store depletion and accordingly, SOCE was not activated. We hypothesized that mitosis-specific phosphorylation of STIM1 may underlie the block of STIM1 rearrangement and SOCE suppression. To this end, the phospho-specific MPM-2 antibody recognized eYFP-STIM1 immunoprecipitated from mitotic but not interphase cells. MPM-2 recognizes phosphorylated serine or threonine followed by proline, and human STIM1 contains 10 instances of S/T-P, all located in the cytoplasmic, C-terminus. STIM1 truncation mutants indicate that at least 2 sites within the C-terminus account for the mitosis-specific phosphorylation. Individual phosphorylation site mutants are being created to identify specific phosphorylated residues and to determine the functional consequences of phosphorylation during mitosis. Suppression of SOCE during mitosis may be an important signaling event, because mitotic processes such as chromosome separation and cytokinesis are exquisitely sensitive to small changes in cytoplasmic Ca^{2+}.

2879-Plat

Heteromeric channel assembly of Orai1 and Orai3 exhibits altered Ca2+ selectivity

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Coexpression of STIM1, targeted to the endoplasmic reticulum and each of the three Orai (also termed CRACM) channels located in the plasma-membrane leads to store-operated, highly Ca2+ selective currents. While Orai1 has been reported to form the native Ca2+ release activated Ca2+ (CRAC) channels in human T-cells, the molecular architecture of less Ca2+ selective store-operated currents remains unknown. Here we show employing confocal fluorescence resonance energy transfer (FRET) that all three Orai proteins are able to form homo- and hetero oligomers. Overexpressed homomeric Orai1 or Orai3 together with STIM1, resulted in store-operated inward rectifying, highly Ca2+ selective currents, as resolved by whole-cell patch-clamp recordings. Coexpression of Orai1 together with Orai3 and STIM1 yielded similar store-depletion activated Ca2+ currents, yet with a leftward shifted reversal potential, pointing to less selective currents. In line, a tandem construct where Orai1was linked to Orai3 exhibited a similarly reduced Ca2+ selectivity that allowed for robust Cs+ permeation. Moreover, Orai3 pore mutants coexpressed with wild-type Orai3 affected Ca2+ and Cs+ selectivity/permeability. These

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,