451-Pos Assessing Cadherin-Cadherin Binding Dynamics in Living Cells Using Förster Resonance Energy Transfer (FRET)

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Board B283

Cadherins are a major family of calcium-dependent cell-cell adhesion molecules that are found at cellular junctions and neuronal synapses. Classic cadherins interact via homotypic adhesion_in a cis-dimer interaction within each membrane as well as a trans-dimer (across junction) interaction between the cis dimers. To monitor directly the binding dynamics, we have developed a Förster Resonance Energy Transfer (FRET) reporter system for visualizing homophilic interactions of cadherin in living cells. We created N-cadherin fusion proteins with an intramolecular fluorescent protein insertion (cerulean, donor and venus, acceptor) using a transposon-mediated insertion method. We generated fluorescently labeled N-cadherin that is functional, exhibits fluorescence of sufficient intensity for FRET, mimics the cellular localization of endogenous N-cadherin, and interacts with the correct binding partner, β-catenin. In heterologous cells, we can probe within cell or across junction cadherin interactions when individual cells are co-transfected and express both constructs (cis) or neighboring cells express either the donor or acceptor (trans). The dynamics of Ca^{2+}-dependent cadherin associations can be monitored by manipulating extracellular Ca^{2+}. Since cadherins play a critical role in synaptic plasticity, we will use the FRET reporter system in neurons to directly test whether synaptic activity regulates the adhesive strength of cadherin interactions across synapses. FRET will be used to detect cadherin interactions between pre- and postsynaptic cells during synaptic activity and varying extracellular Ca^{2+}concentrations. These experiments will test our hypothesis that cadherins act as extracellular calcium detection system to coordinate synaptic plasticity across the synapse.

Calcium Signaling Pathways

452-Pos Ca^{2+} Store Depletion Triggers A Dynamic, Physical Coupling Of Stim1 To Orai1

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Board B284

Store-operated Ca^{2+} entry (SOCE) is a physiologically important process that is triggered by the depletion of intracellular Ca^{2+} stores. Recently, STIM1 an endoplasmic reticulum (ER) protein and ORAI1, which is thought to act as the channel unit in the plasma membrane (PM), were identified as essential components of the classical calcium-release-activated calcium (CRAC) current. STIM1 with an EF hand motif in the lumen of the ER, senses the Ca^{2+} concentration and redistributes after store depletion into punctae in regions tight beneath the PM. Here we demonstrate by confocal Förster Resonance Energy Transfer (FRET) microscopy that this redistribution results in a dynamic, physical coupling of STIM1 to ORAI1 that culminates in the activation of Ca^{2+} entry. FRET imaging of living cells provided insight in the time-dependence of crucial events of this signalling pathway comprising Ca^{2+} store depletion, STIM1 multimerization and STIM1-ORAI1 interaction and furthermore resolved a significant time lag between STIM1-STIM1 and STIM1-ORAI1 interactions. Store refilling reduced both STIM1 multimerization as well as STIM1-ORAI1 interaction suggesting a reversible process, which is specifically linked to the loading state of the ER. The cytosolic STIM1 C-terminus itself was able, in vitro as well as in vivo, to physically associate with ORAI1 and to stimulate channel function without store depletion indicating a crucial role of this domain in the activation mechanism. Our experiments do not exclude that CRAC current activation may well occur with the help of auxiliary components. Further elucidation of such components involved in fine tuning of the STIM1-ORAI1 coupling process will hopefully widen the repertoire for manipulation of this key mechanism by the development of both specific inhibitors as well as activators.

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453-Pos Shear-stress Induced Ca^{2+} Transients in Ventricular Myocytes are Associated With Rapid Decline of the Mitochondrial Ca^{2+} Content

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In cardiomyocytes, we have found that brief "puffs" of solution (shear-stress) trigger cytosolic Ca^{2+}-transients that are independent of SR Ca^{2+} release and display pharmacology indicative of a mitochondrial origin. To investigate mitochondrial Ca^{2+}-signaling, we used rapid confocal fluorescence imaging of cells stained with the potentiometric dye, TMRE or the Ca^{2+}-sensitive dye, rhod-2. The images showed the mitochondria as longitudinal lines with a separation of 1-2 μm and a regular striation pattern at ~2 μm. During electrical stimulation, KCl-depolarization, "puffs", and exposure to caffeine or mitochondrial blockers (FCCP or CCCP), the images revealed not only a change in the overall fluorescence intensity, but also redistribution between the mitochondrial and cytoplasmic compartments. To eliminate contraction artifacts, we analyzed the images based on their Fourier-transforms, where the typical mitochondrial pattern was represented by transverse and sarcomeric harmonics. Changes in the TMRE-images displayed different time-course for average fluorescence intensity vs. harmonics, suggesting that this dye increases its brightness when redistributed from the

mitochondrial to the cytoplasmic compartment. Similarly, the rhod-2 signals suggested two components, one of which was highly variable and appeared to reflect cytosolic Ca^{2+}. We permeabilized the myocytes to eliminate this cytosolic component and the signal remaining under these conditions was consistent with measurements from a single mitochondrial compartment. Thus, the decline in TMRE-fluorescence evoked by FCCP was similar for average fluorescence and different harmonics. In turn, rhod-2 provided a truer measurement of mitochondrial Ca^{2+}, which was enhanced during exposure to caffeine, rapidly reduced by "puffs" and slowly depleted by CCCP. These results demonstrate a labile mitochondrial Ca^{2+}-pool that can be pre-loaded during caffeine-induced Ca^{2+} release from the SR and rapidly released by "puffs". The mechanism by which mechanical forces signal mitochondrial Ca^{2+}-release remains unknown.

454-Pos The Role Of Store Operated Calcium Entry (SOCE) In Human Neuroendocrine Tumors

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Board B286

Gastroenteropancreatic neuroendocrine tumors (GEPNETs) are slow-growing cancers originating primarily from amine or peptide secreting enteroendocrine cells of gastrointestinal tract. Substantial evidence suggests an inverse relationship between calcium intake and colorectal cancer. However, little is known regarding the role of luminal or cytosolic calcium on GEPNETs of small intestine and bowel. Initial experiments demonstrated that, in contrast to a non-cancer intestinal epithelial cell line, exposing carcinoid cell lines to elevated extra-cellular calcium levels (5mM) enhanced cell growth. Calcium entry via voltage-dependant calcium channel (VDCC) and store-operated calcium channels (SOCC) play vital roles in secretion, proliferation and apoptosis in a variety of cell types. Yet, we are far from understanding the role of store-operated calcium entry (SOCE) in GEPNETs. In this study, calcium imaging and cell proliferation assays were used to investigate the function of SOCCs in human foregut, midgut and hindgut carcinoid cell lines. All cell lines exhibited SOCE. In addition, treatment with SOCE blockers diminished calcium influx in the carcinoid cell lines, and reduced cell proliferation determined by MTT assay and flow cytometry. We then used RT-PCR to identify SOCE related transcripts in human carcinoid cell lines, including Trpc1-7, Trpv5&6, Orai 1–3 and Stim1. We demonstrate that carcinoid cell lines express various SOCE genes of which Trpc1, the transcripts for the recently discovered calcium channels, Orai1, and calcium sensor protein STIM1 were strongly expressed in these carcinoid cell lines. Current studies are correlating the role of these genes in calcium influx and their role in cell growth and proliferation in carcinoid cells. Thus, targeting SOCCs may pave the way for treatment of carcinoid tumors where current therapies are not effective.

455-Pos Chromogranin B impacts calcium dependent and NF-λB mediated cardiac hypertrophy

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Altered regulation of signaling pathways can lead to pathological conditions including cardiac hypertrophy and heart failure (HF). We report that neonatal and adult cardiomyocytes express chromogranin B (CGB), a calcium (Ca^{2+}) binding protein which modulates Ca^{2+} release by the inositol 1,4,5-trisphosphate receptor ($InsP_3R$). Immunocytochemistry showed co-localization of CGB and the $InsP_3R$ type 2, the predominant $InsP_3R$ isoform expressed in cardiomyocytes. Using fluorescent Ca^{2+} indicator dyes, we found that CGB regulates $InsP_3$-dependent Ca^{2+} release in response to angiotensin-II (ANG-II), an octapeptide hormone that promotes cardiac hypertrophy. ELISA experiments and luciferase reporter assays identified ANG-II as a potent inducer of brain natriuretic peptide (BNP), a hormone that recently emerged as an important biomarker in cardiovascular disease. CGB was also found to regulate ANG-II stimulated and basal secretion, expression and promoter activity of BNP that depend on the $InsP_3R$. Moreover, we provide evidence that CGB acts via the transcription factor nuclear factor-kappa B (NF-κB) in an $InsP_3/Ca^{2+}$-dependent manner. Remarkably, this process is independent of nuclear factor of activated T-cells (NFAT). *In-vivo* experiments further showed that ANG-II induced cardiac hypertrophy, a condition characterized by increased ventricular BNP production, is associated with an extensive up-regulation of ventricular CGB expression. Over-expression of CGB in cardiomyocytes in turn induced the BNP promoter. The evidence presented in this study identifies CGB as a novel regulator in Ca^{2+}/$InsP_3$-dependent cardiac hypertrophy. This modulator of the signaling pathway might become a new therapeutic target for patients with cardiovascular disease, including cardiac hypertrophy and HF.

456-Pos CaMKII Activation Links Hypertension to Cardiac Hypertrophy and Heart Failure

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Hypertension is a major risk factor for developing cardiac hypertrophy and heart failure (HF). However, the molecular mechanism linking hypertension to heart disease is incompletely understood. We hypothesized that alterations in the Ca^{2+}-calmodulin-CaMKII signaling pathway are fundamental in the development of HHD. To test this hypothesis, we examine the CaMKIIδ protein expression

and phosphorylation in the left ventricular tissue at progressive stages of HHD development: ***pre-hypertension, hypertension, cardiac hypertrophy*** and ***HF***, using the spontaneously hypertensive rat (SHR) model. In order to separate HHD-related changes from aging-related changes, we also conducted a longitudinal study using age-matched Wistar-Kyoto rats (WKY).

RESULTS:

1. CaMKIIδ phosphorylation at the Thr287 site was significantly elevated in SHR (two-way ANOVA test, $p<0.0001$ for the difference between SHR and WKY). The onset of hypertension in SHR was associated with a marked increase of CaMKIIδ-Thr287 phosphorylation indicative of increased CaMKII activity; this hyperphosphorylation persisted throughout the development of cardiac hypertrophy and HF under essential hypertension.
2. The protein expression levels of δ_B (nuclear) and δ_C (cytosolic) isoforms of CaMKIIδ are the same in SHR and age-matched WKY, showing no HHD-related changes, although there are aging-related changes in the protein expression and phosphorylation of δ_B and δ_C in both SHR and WKY groups.
3. Using angiotensin converting enzyme (ACE) inhibitor, enalapril to prevent the onset of hypertension in SHR resulted in reduction of the CaMKII-Thr287 phosphorylation, strongly supporting a causal relationship between hypertension and CaMKII activation.

CONCLUSION: The data show that CaMKIIδ signaling pathway is activated by hypertensive stress. Given the known effects of CaMKIIδ on inducing gene transcription and E-C coupling remodeling, we suggest CaMKIIδ plays a significant role in linking hypertension to the development of cardiac hypertrophy and HF.

457-Pos Computational Study of Ca2+ Oscillations and Diacylglycerol Signals on activation of Protein Kinase C

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Activated phospholipase C (PLC) generates 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) from phosphatidyl inositol (PIP_2). The DAG remains in the plasma membrane and co-activates conventional protein kinase C (cPKC) with Ca^{2+}. Two computational models were developed to study how Ca^{2+}, IP_3, DAG, PKC, and PLC interact to produce experimentally observed dynamics. Both models include activation of PKC by Ca^{2+} to inhibit PLC cleavage of PIP_2. Experiments suggest that for some isoforms of PLC, there might be fast activation of PLC by Ca^{2+}. The first model demonstrates that fast positive feedback on PLC is not necessary to explain the experimental observation of Ca^{2+} and DAG dynamics for cells that use primarily PLCγ, such as the T lymphocyte. The model suggests that oscillations in Ca^{2+} and DAG are possible without fast positive Ca^{2+} feedback on phospholipase C. In this model, Ca^{2+} oscillations are observed with inhibition of the PKC feedback on PLC or addition of non-degradable IP_3, suggesting that oscillations in IP_3 are not necessary for Ca^{2+} oscillations. A second proposed model was developed to study how the fast positive feedback of Ca^{2+} on PLCβ affects Ca^{2+}, IP_3 and DAG dynamics. The model is able to generate calcium oscillations induced by changes in IP_3 production with PKC inhibition with a slight shift in the bifurcation diagrams for Ca^{2+} oscillations. The models suggest that Ca^{2+}/calmodulin-binding PLCβ can modulate the frequency of oscillating Ca^{2+} by changes in IP_3 production, but Ca^{2+} increased activation of PLCβ is not required for driving Ca^{2+} oscillations.

458-Pos CRAC Channels in Developing Human Dendritic Cells

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According to their phenotype, function, and tissue localization two types of dendritic cells (DCs) can be distinguished. Immature DCs (IDCs) make the first contact with foreign antigens in the periphery (e.g. skin) where they take up and process them. When an infection occurs, they are stimulated to migrate to the local lymphoid tissues, where they have a completely different, "mature" phenotype (MDCs); the expression of MHC and other co-stimulatory molecules enables them to stably present peptides from proteins acquired from the infecting pathogens. Among other signaling pathways the role of the cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) in the differentiation and function of DCs is recognized. To date, however, little is known about the mechanisms being responsible for the rise in the $[Ca^{2+}]_i$ in human DCs.

Human dendritic cells at different stages of differentiation were produced *in vitro* from freshly isolated human monocytes. The $[Ca^{2+}]_i$ was measured using FURA-2 in ratiometric mode of a PTI Delta Scan microscope fluorimeter. Ionic currents through CRAC channels were measured in the whole-cell mode of patch-clamp. The pipette filling solution was based on Cs-methansulfonate supplemented with 10 mM Mg^{2+} to inhibit MIC currents.

Recording of ionic currents in voltage clamped IDCs and MDCs failed to identify any voltage-gated Ca^{2+} conductance. Depletion of the intracellular Ca^{2+} stores using thapsigargin (1 μM) evoked a biphasic Ca^{2+} signal in both IDCs and MDCs. Ion substitution experiments showed that the second phase of the signal is absent 0 mM extracellular Ca^{2+} characteristic of the activation of CRAC channels. Pharmacological experiments using CRAC blockers SKF-96365 (50 μM) and 2-APB (40 μM) inhibited the Ca^{2+} signal. These experiments pointed to CRAC channels as the primary channels being responsible for Ca^{2+} signaling in DCs. These findings were confirmed using patch-clamp.

459-Pos

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No Abstract

460-Pos IP_3 and RyR Ca^{2+} Signaling in Ca^{2+} Buffered Rat Neonatal and Juvenile Cardiomyocytes

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Ca^{2+} triggered Ca^{2+} release from ryanodine receptors (RyR) is the major Ca^{2+} signaling mechanism in adult hearts, but it is less clear how dominant this mechanism is in neonatal hearts. Previously we have shown the presence of RyR and IP3-gated pools in intact and permeabilized neonatal day 1–2 and juvenile day 8-10 rat cardiomyocytes. Here we used dialysis of Ca^{2+} buffers (EGTA) into voltage clamped neonatal and juvenile cardiomyocytes to analyze the spatial and temporal Ca^{2+} signaling patterns upon activation of RyR or IP_3. Hearts of neonatal and juvenile rats were removed and digested with collagenase and protease. The resulting cardiomyocytes were plated, voltage clamped and dialyzed with EGTA and fluo-4, and then imaged confocally at 120 frames/s. These cardiomyocytes were then probed for the presence of RyR and IP_3 induced Ca^{2+} signals using alternate exposures to caffeine or ATP. The spatial caffeine induced Ca^{2+} release pattern was similar in both age groups, with the signal beginning at the periphery of the cell and spreading rapidly throughout the cell. Temporal analysis revealed that the time to peak response was faster in the juvenile cardiomyocytes. The spatial and temporal ATP induced Ca^{2+} signal pattern, which was similar in the two age groups, was uniquely different from that of caffeine, revealing slow membrane Ca^{2+} spark oscillations, followed by frequent localized internal sparks and occasional delayed Ca^{2+} transients. We conclude that ATP induced Ca^{2+} signaling has a unique Ca^{2+} release pattern from that of caffeine in developing cardiomyocytes. Also, interestingly, IP_3 activation can trigger Ca^{2+} transients in Ca^{2+} buffered neonatal and juvenile cardiomyocytes, perhaps indicating a role for IP3 in Ca^{2+} signaling in the developing heart.

461-Pos Abnormal Mitochondria And Ca^{2+} Signaling In The Skeletal Muscle Of A Murine Model Of Amyotrophic Lateral Sclerosis

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Amyotrophic lateral sclerosis (ALS) is a fatal neuromuscular disorder involving degeneration of motor neurons and atrophy of skeletal muscle. Mutations of superoxide dismutase (SOD1) are associated with 20% of inherited ALS cases. Transgenic mice expressing human ALS-linked mutation in SOD1 (G93A) develop an ALS-like phenotype (Gurney 1994). We study this mouse model seeking to understand the cellular mechanisms that underlie muscle dysfunction in ALS. We characterized simultaneously mitochondrial structure and function in enzymatically dissociated muscle fibers loaded with MitoTracker Deep Red and TMRE, a probe of mitochondrial membrane potential. Deficient TMRE staining revealed loss of potential in fiber segments of ALS cells. The same segments showed fuzzy staining by MitoTracker, with loss of contrast, indicating alterations in mitochondrial shape in the same damaged region. By contrast, the two dyes stained the wild type fiber equally and homogeneously. Electron microscopy (EM) also revealed defective mitochondria in the ALS muscle, with intrusion of odd-shaped mitochondria into the sarcomeric A band, in contrast with the normal shape and restriction to the I band of mitochondria in controls. Preliminary images of Ca^{2+} events induced by osmotic shock (Wang 2005) in cells dually stained with fluo-4 and TMRE showed that regions with defective mitochondria had 6-fold (n=3) higher frequency of Ca^{2+} sparks. This result suggests that mitochondrial malfunction is accompanied by abnormal Ca^{2+} signaling in ALS muscle.

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462-Pos Expressional and Functional Switch of Ca^{2+} Release Receptors in Developmental Cardiomyocytes

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Ca2+ release receptors/channels are crucial for cell function, and they are also very important for development of tissues and organs. To investigate the possible changes and the role of Ca2+ release receptors/channels in developmental cardiac myocytes, the expression and function of the these receptors as well as ultrastructure of developing cardiomyocytes were examined on daily bases after 7.5 embryo day (E7.5d) by using multiple approaches. The main findings of the present study include 1) in cardiac myocytes day 7.5~10.5 of embryos both ryanodine and IP3 receptors are expressed, but at this time the E-C coupling is still not completely established which is supported by patch-clamp and confocol microscope combined study in which normal Ca2+ sparks could not be observed, but Ca2+ waves with IP3R release property observed frequently; 2) after day 10.5 the expression and function of RYRs increase while the IP3R decrease with development, and the Ca2+ induced Ca2+ release (CICR) established; 3) ultra structure study indicates that in early developmental stage (before E10.5d) the coupling between cell membrane and sarcoplasmic reticulum (SR) has not been efficient, and distribution of rough endoplasmic reticulum(RES) is abundant; 4) whole heart image study in vivo demonstrates that the propagation speed of Ca2+ singling displays a time delay between atrium and ventricle before E10.5d (that is “tube stage”); 5) action potential property exhibits a long duration and a fast velocity in early cardiac developmental stage. These results suggest that a functional and structural switch of Ca2+ release receptors/channels and cardiomyocytes exists during heart development.

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463-Pos Inhibition Of α-calcium/calmodulin-dependent Protein Kinase II (αCaMKII) Upon Ca^{2+} Removal By Sequential Domain Dissocation Of CaM

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αCaMKII is implicated in learning and memory by means of long-term potentiation. Activation of αCaMKII by Ca^{2+}/calmodulin (Ca^{2+}/CaM) results in rapid Thr286-autophosphorylation. Upon Ca^{2+} removal, phospho-Thr_{286}-αCaMKII remains partially active as measured at 30°C for 2 minutes and it is then inactivated by a burst of $Thr_{305/306}$-autophosphorylation. Ca^{2+}/CaM dissociation is assumed in the process but has not been demonstrated[1(review)]. We set out to determine whether the inactivation, CaM dissociation or $Thr_{305/306}$-autophosphorylation is the rate-limiting step in the Ca^{2+} removal induced inactivation of phospho-Thr_{286}-αCaMKII. Kinase activity was measured using by a continuous steady-state coupled enzyme reaction[2] with syntide-2 as substrate. Inactivation was induced by the addition of 4.2 mM EGTA. CaM conformational change was observed with DA-CaM[3] and CaM dissociation was measured by polarisation of FL-CaM. $Thr_{305/306}$-autophosphorylation was quantified by western blotting. Temperature dependence of αCaMKII activity and EGTA-induced inactivation showed that the activation energy E_a of the two processes was similar, 101 and 106 kJ/mole respectively. At 37 °C DA-CaM showed a biphasic conformational change (k_1 0.83 s^{-1}, A1 0.70; k_2 0.088 s^{-1}, A2 0.3) corresponding to a compact to extended transition suggesting that CaM may have been attached by a single lobe. Depolarisation of FL-CaM indicated complete dissociation occurring at 0.024 ± 0.016 s^{-1}. EGTA induced inactivation occurred in an exponential process with a rate of 0.0125 ± 0.0001 s^{-1} and $Thr_{305/306}$-autophosphorylation proceeded at a rate of 0.0124 ± 0.0037 s^{-1}. We propose that Ca^{2+} removal induces the sequential domain dissociation of CaM and thereby a time-dependent inhibition of phospho-Thr_{286}-α CaMKII.

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464-Pos Functional Grouping of EF-Hand Proteins by Comparison of Interhelical Essential Dynamics

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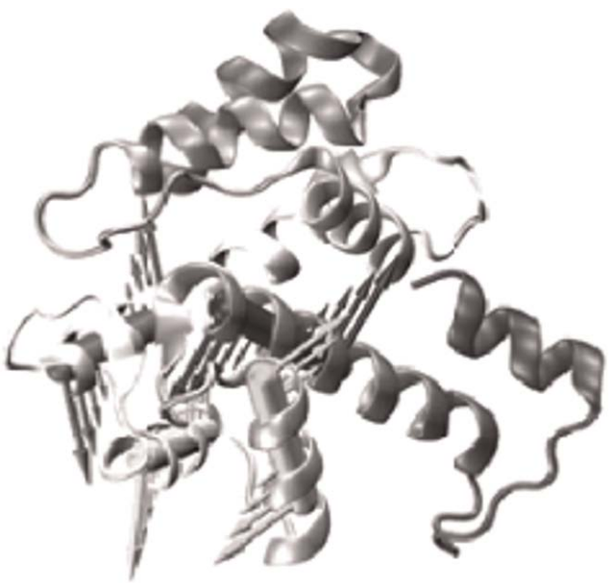
EF-Hand calcium binding proteins are among the major actors in signal transduction. This functionality relies on the plasticity of the EF-hand domains whose constituting helices undergo major rearrangements upon calcium binding. The elastic properties underlying the concerted large scale motion of the helices are here investigated, by means of a coarse-grained model, for a comprehensive set of representatives of this protein superfamily[1,2]. For each representative, the large scale movements are described in terms of collective degrees of freedom which correspond to the six interhelical angles formed by the four helices in each EF-hand domain. This choice of degrees of freedom provides a common framework for comparing the concerted large scale dynamics among all representatives. It is found that, despite the structural heterogeneity of the set [3], only a very limited number of possible interhelical movements exist[1]. This feature is exploited to subdivide the calcium-binding protein representatives in four groups, reflecting the similarity in interhelical dynamics.

The possible biological implication of the results are discussed by comparing the dynamics-based grouping with previously-introduced ones based on structural features alone.



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465-Pos The Kinetics Of Ca^{2+}-dependent Switching In A Calmodulin-IQ Domain Complex

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We have performed a kinetic analysis of Ca^{2+}-dependent switching in the complex between calmodulin (CaM) and the IQ domain from neuromodulin, and have developed detailed kinetic models for this process. Our results indicate that the thermodynamically complementary effects of the IQ domain on Ca^{2+}-binding to the C-terminal and N-terminal CaM lobes are due to:

1. A ~10-fold decrease in the association rate for the C-terminal Ca^{2+}-binding sites and
2. A ~3-fold decrease in the dissociation rate for the N-terminal sites.

The transition from Ca^{2+}-free to Ca^{2+}-saturated complex can occur with Ca^{2+} binding initially to either the N-terminal or C-terminal lobe, followed by binding to the remaining sites. Partitioning between these kinetically distinct pathways is dependent upon the amplitude and speed of the Ca^{2+} concentration increase. The transition from Ca^{2+}-saturated to Ca^{2+}-free complex occurs in an sequential manner, beginning with Ca^{2+} dissociation from the N-terminal lobe. Although the Ca^{2+}-free and Ca^{2+}-saturated forms of the CaM-IQ domain complex have identical affinities, CaM associates and dissociates ~100 times faster in the presence of Ca^{2+}. Furthermore, Ca^{2+}-saturated CaM can be transferred at an accelerated rate to the CaM-binding domain from CaM kinase II via a ternary complex. These properties are consistent with the hypothesis that CaM bound to neuromodulin comprises a localized store that can be efficiently delivered to neuronal proteins in its Ca^{2+}-bound form in response to a Ca^{2+} signal.

466-Pos Effects Of A Novel NAADP Antagonist On Cell Contractions And Calcium Transients In Guinea-pig Isolated Ventricular Myocytes

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Nicotinic acid adenine dinucleotide phosphate (NAADP) is a novel endogenous mediator thought to mobilize Ca^{2+} from acidic organelles. We have reported previously that NAADP increased whole cell Ca^{2+} transients and contractions in guinea-pig ventricular myocytes, when photoreleased from a caged analogue. Furthermore, application of the cell membrane-permeant acetoxymethyl ester of NAADP (NAADP-AM) increased contraction amplitude of guinea-pig ventricular myocytes and the frequency and amplitude of Ca^{2+} sparks in rat myocytes. Here we present observations with Ned-14, a novel, cell membrane-permeant NAADP antagonist which has been shown to abolish NAADP-mediated Ca^{2+} release, but not IP_3 or cADPR-mediated Ca^{2+} release, in the sea urchin egg homogenate preparation. Guinea-pig ventricular myocytes were electrically field stimulated (3 ms pulses, 1 Hz; 36 °C). Contractions were measured using an edge detection system. Ca^{2+} transients were imaged using line-scan confocal microscopy (fluo-5F). Rapid application of Ned-14 caused concentration-dependent decreases in myocyte contraction. Following 30 s application, Ned-14 reduced contraction by 35±6 % at 100 μM (n=5, $p<0.01$) and by 19±3 % at 30 μM (n=9, $p<0.01$); no significant changes were observed with 10 μM Ned-14 (2±4 %, n=8). Ned-14 (100 μM) also decreased Ca^{2+} transient amplitude and decay half time (−22±3 % and −23±5 % at 30 s, n=6). These data are consistent with antagonism of the actions of endogenous NAADP. In further experiments, Ned-14 was found to inhibit the effects of NAADP-AM (60 nM). Application of NAADP-AM alone caused an increase in contraction amplitude of 37±6 % at 20 s (n=10, $p<0.01$), compared to a 8±3 % change (n=5, $p>0.05$) in the presence of 100 μM Ned-14. Ned-14 may present a novel tool with which to investigate the role of NAADP in intracellular Ca^{2+} signalling.

467-Pos Succinate regulates cardiomyocyte function

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Succinate is a mediator of the Citric Acid Cycle whose receptor, GPR91, was recently characterized and identified in several tissues such as kidney, liver and spleen. In these tissues, it has been observed that the linking of succinate to its receptor triggers an increase of intracellular Ca^{2+}. In the heart, the role of succinate is not yet known. Our goal was to investigate the role of succinate in cardiomyocytes using a combination of molecular biology, and Ca^{2+} imaging techniques. We found that cardiomyocytes express GPR91 in the plasma membrane. Moreover, succinate (10 mM) treatment of cardiomyocytes increases peak Ca^{2+} transient. Ca^{2+} transient kinetics of decay were also altered in succinate treated cardiomyocytes. Ca^{2+} re-uptake to the sarcoplasmatic reticulum was faster in cardiomyocytes treated with succinate when compared to control cardiomyocytes. This change can be rationalized by an increase in phospholamban phosphorylation levels in succinate treated cells. Increased ryanodine receptor phosphorylation levels were also observed after succinate treatment. These findings suggest that succinate has an important role in cardiomyocytes function.

468-Pos Formation Of Large Endocytic And Non-endocytic Vacuoles In Pancreatic Acinar Cells

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Using confocal and two-photon microscopy as well as fluorescent-labelled dextrans added to extracellular solution we monitored formation of endocytic vacuoles in cells stimulated by supramaximal concentrations of calcium-releasing secretagogues. We observed formation of large endocytic vacuoles (1–6μm in diameter) in apical (secretory) part of the cell followed by translocation of these vacuoles to lateral and basal regions. Activation of trypsinogen

was observed in some of these giant vacuoles. Formation of other type of vacuoles was observed using infusion of fluorescent-labelled dextrans into the cytosol of the cell. In these experiments fluorescence labelled dextrans served as contrasting indicators. Finally, combination of two probes (intracellular and extracellular) allowed us to visualize both endocytic and non-endocytic vacuoles.

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469-Pos Trpc6 And Kv7.5 Channel Contributions To Resting Membrane Potential And Effects Of Physiological Vasopressin Concentration In A7r5 Vascular Smooth Muscle Cells

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Physiological concentrations of [Arg^8]-vasopressin (AVP, 100 pM) induce membrane depolarization and stimulate action potential firing in A7r5 vascular smooth muscle cells. We have previously implicated suppression of Kv7.5 voltage-dependent potassium (K_v) currents as a depolarizing stimulus, but non-selective cation currents (I_{CAT}) may also contribute. TRPC6 channels are non-selective cation channels that are activated by AVP. Using whole-cell perforated patch clamp techniques we measured K_V currents, I_{CAT}, and membrane potential in A7r5 cells. Kv7.5 and TRPC6 expression levels were suppressed by treatment with short hairpin RNA constructs (shRNAs). K_v currents measured at −20 mV were reduced by 99% in Kv7.5 shRNA-expressing cells (n=7), but were not affected by treatment with TRPC6 shRNA (the latter treatment reduced I_{CAT} by 70% (n=11)). L-type calcium current densities were not significantly different among the control or either shRNA treated cells. Expression of Kv7.5 shRNA resulted in significantly more positive resting membrane potential: −16±4.1 mV (n=9) in transfected cells versus −51.6±1.9 mV in control cells (n=6). Application of 100 pM AVP induced greater depolarization in control (9.8±1.2 mV, n=6) than in Kv7.5 shRNA-treated cells (5.9±1.1 mV, n=9). In contrast, neither resting potential nor the level of AVP-induced depolarization was altered by TRPC6 shRNA expression. These results suggest that:

1. Kv7.5 potassium channels are necessary for setting resting membrane potential in A7r5 vascular smooth muscle cells;
2. Inhibition of Kv7.5 potassium current is an essential step in AVP-induced membrane depolarization;
3. Activation of TRPC6-mediated non-selective cation current does not contribute significantly to the depolarization induced by physiological [AVP].

470-Pos UVA-induced Cytosolic Calcium Oscillations in Mast Cells: a Dominant Role for NADPH Oxidase-Derived Reactive Oxygen Species

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Board B302

Phototherapy or ultraviolet (UV) irradiation therapy has been in use for many years in the clinic. UVA penetrates skin tissue deeper than UVB, to reach the dermal layers and therefore affect sub-dermal cells such as dermal fibroblasts, endothelial cells, T lymphocytes, and mast cells. UVA irradiation leads to immunosuppressive and anti-inflammatory effects, but the detailed molecular mechanisms are not fully understood. We in this work have investigated UVA irradiation effects on isolated mast cells. It was found that UVA induced regular cytosolic calcium oscillations in rat mast cells. The regular calcium oscillations induced by UVA was inhibited completely by PI-specific phospholipase C (PLC) inhibitor U73122, whereas U73343, a structural analog, was without any effect. Tetrandrine, a Ca^{2+} channel antagonist, or external Ca^{2+}-free buffer abolished oscillations induced by UVA. Nifedipine, the L-type calcium channel inhibitor, and SK&F96365, was without any effect on UVA-induced calcium oscillations. UVA irradiation induced marked increase in ROS levels; after pre-treatment of mast cells with the ROS quencher NAC, or with the NADPH oxidase inhibitor DPI, such ROS increases were no longer seen; accordingly no calcium oscillations were recorded from these treated cells any more. It is concluded that UVA induces $[Ca^{2+}]_i$ oscillations by activating the PLC pathway after generation of intracellular ROS. Calcium oscillations can then modulate additional cellular functions such as gene expression.

Keywords

mast cell, UVA, ROS, calcium oscillation, NADPH oxidase

471-Pos PKC epsilon Regulates Calcium Sparks By Selectively Interacting With Type-1 Ryanodine Receptors In Smooth Muscle Cells

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Protein kinase C (PKC) can regulate Ca^{2+} sparks in vascular and airway smooth muscle cells (SMCs), but its specific molecular mechanisms remain elusive. In this study, we aimed to determine whether PKCε may regulate Ca^{2+} sparks by interacting with ryanodine receptors (RyRs) and which subtype of RyRs underscores the effect of PKCε in SMCs. Our data indicate that in airway SMCs, inhibition of PKCε by a specific inhibitory peptide or gene deletion significantly increased the frequency of Ca^{2+} sparks, and decreased the amplitude of Ca^{2+} sparks in the presence of xestospogin-C to eliminate functional inositol 1,4,5-triphosphate receptors. PKCε activation with phorbol-12-myristate-13-acetate (PMA) caused a significant decrease in Ca^{2+} spark frequency and increase in Ca^{2+} spark amplitude in the presence of xestospongin-C. The effect of PMA was completely blocked in PKCε$^{-/-}$ cells. RyR1 gene deletion abolished PKCε inhibition-induced increase in Ca^{2+} spark frequency and decrease in Ca^{2+} spark amplitude. The effect of PKCε activation was also prevented in RyR1$^{-/-}$ cells. Modification of RyR2 activity by FK506-binding protein 12.6 gene deletion did not annihilate the effect of PKCε inhibition and activation on either Ca^{2+} spark frequency or amplitude. PKCε inhibition-elicited increase in Ca^{2+} spark frequency and decrease in Ca^{2+} spark amplitude was not eliminated in RyR3$^{-/-}$ cells. RyR3 gene deletion did not inhibit the effect of PKCε activation on Ca^{2+} sparks, either. In conclusion, PKCε regulates Ca^{2+} sparks by specifically interacting with RyR1 in airway myocytes. This novel mechanism to regulate Ca^{2+} sparks may have a physiological importance in SMCs.

Calcium Signaling Proteins

472-Pos Developing Calcium and Proteinase Sensors for Real-time Imaging

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We demonstrate the successful design of metal-binding site in several non-metal-binding proteins with desired metal selectivity. More interestingly, these designed proteins retain their native ability to associate with natural target molecules. The solution structure reveals that designed metal binding proteins bind metal ions at the intended site with the designed arrangement, which validates our general strategy for designing de novo metal-binding proteins with multiple functionalities. The structural information also provides a close view of structural determinants that are necessary for a functional protein to accommodate the metal-binding site. Using our design approach, we have developed several fluorescent protein-based sensors with a wide range of affinities that can be applied to monitor calcium signaling at different cellular environments and disease pathways. Different from other available sensors, our developed calcium probes have unique advantages as they do not alter the natural calcium signaling network. In addition, sensors for several different classes of proteinases, such as caspases, thrombin, trypsin, chymotrypsin, have also been developed for real-time imaging. These developed ratiometric sensors are comprised of a single fluorescent protein in contrast to other FRET based sensors which utilized paired fluorescent proteins. They are specifically ideal for monitoring cellular responses at different compartments and quantitative analysis.

Calcium Fluxes, Sparks and Waves

473-Pos Photo-Control of Calmodulin Binding to Target Peptide using Photochromic Compound

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Board B305

Calmodulin (CaM) is a physiologically important Ca^{2+}-binding protein that participates in numerous cellular regulatory processes. CaM has a dumbbell-like shape in which two globular domains are connected by a short α-helix. Each of the globular domains has two Ca^{2+}-binding site called as EF-hand. CaM undergoes a conformational change upon binding to calcium, which enables it to bind to specific proteins for a specific response. In this study, we have demonstrated that photo-control of CaM binding to target peptide using photochromic compound N- (4-phenylazophenyl) maleimide (PAM) which undergoes *cis-trans* isomerization by ultraviolet (UV) - visible (VIS) light irradiation reversibly. PAM was incorporated into CaM mutants that have a single reactive cysteine residue. And we prepared fluorescent fusion protein M13-YFP in order to monitor interaction between CaM and M13 peptide with HPLC using size exclusion column. The binding of PAM-CaM (N60C), PAM-CaM (D64C) and PAM-CaM (M124C) to M13-YFP were apparently photo-controlled by UV-Visible light irradiation reversibly at the appropriate Ca^{2+} concentration. Interestingly, on UV light irradiation, the binding of PAM-CaM (N60C) and PAM-CaM (D64C) increased. Contrary, the binding of PAM-CaM (M124C) was decreased. And on VIS light irradiation, the binding of the PAM-CaM mutants showed opposite effect to UV light irradiation. Currently, we are trying to regulate CaM dependent enzymes using the PAM-CaM reversibly by UV-VIS light irradiation.