into how LTCs are coupled to cytoskeletal signaling pathways in neurons and shed light on the molecular mechanisms underlying the generation of TS and other Autism Spectrum Disorders.
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1144-Plat
End-stage Mechanisms Underlying Voltage and Ca^{2+}/Calmodulin-Dependent Inactivation (VDI and CDI) of Ca_V1.3 Channels
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The past decade has witnessed many discoveries about the early events that underlie calmodulin (CaM) regulation of Ca^{2+} channels. Much is known about the positioning of apoCaM (Ca^{2+}-free) on channels, and the initial Ca^{2+}/CaM interaction sites. Beyond this, precious little is clear about the eventual actions of this central genre of Ca^{2+} channel modulation. Does CDI involve hinged-lid occlusion, selectivity filter collapse, or allosteric inhibition of activation gating? Do CDI and VDI reach the same ultimate conformation? All these proposals remain in flux. Here, we deduce that mutations within the S6 activation gates would produce discriminating effects on activation and inactivation, depending on which mechanism holds true. For the first two end-stage mechanisms (hinged-lid occlusion and selectivity filter collapse), S6 mutations that enhance channel opening are predicted to strengthen channel inactivation. By contrast, for an allosteric mechanism, such mutations would actually weaken inactivation. These predictions motivated exhaustive mutagenesis of the S6 segments of all four homologous domains of Ca_V1.3. We find that S6 mutations affect VDI and CDI in strikingly different ways, indicating a fundamental divergence of their end-stage mechanisms. The pattern seen for CDI agrees remarkably well with that predicted for an allosteric mechanism. By contrast, VDI effects cannot be fully explained by any previously described end-stage mechanism. Instead, mapping the functional VDI effects onto a structural homology model of Ca_V1.3 reveals a telling structural pattern, suggestive of a novel 'hinged-lid-shield' mechanism. In this scheme, Ca_V1.3 channels feature a specialized distal S6 'shield' that repels lid closure. We validate this proposal with experiments in which the integrity of the shield and the mobility of the hinge-lid are independently modified. In all, these advances furnish a rich mechanistic backdrop for the many Ca^{2+} channelopathies involving S6 domains.

1145-Plat
Single Channel Conductance Of CaV2.2 At Physiological $[Ca^{2+}]_{Ext}$
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Ca^{2+} that enters through voltage-gated CaV2.2 channels and binds to a calcium sensor at the transmitter release site links membrane depolarization to activation of synaptic vesicle discharge. Recent evidence supports the hypothesis that the release site calcium sensor is within the single CaV2.2 channel domain. Thus, modeling presynaptic nanophysiology requires knowledge of the channel transport rate at physiological $[Ca^{2+}]_{ext}$. However, this value has only been determined previously for the non-presynaptic CaV1.x (L type) channel with a conductance of ~2.4 pS at $[Ca^{2+}]_{ext}$=2 mM (Church and Stanley, JP 1996). Since at $[Ba^{2+}]_{ext}$=100 mM CaV1.x has a conductance of ~24 pS while CaV2.2 has one of ~14 pS we predicted that at $[Ca^{2+}]_{ext}$=2 mM the latter channel would have a conductance of ~1.2 pS.
Single calcium channels were recorded using low noise, quartz electrodes from freshly isolated chick dorsal root ganglion neurons which express virtually entirely CaV2.2 current. In the presence of $[Ca^{2+}]_{ext}$=2 mM and 2 μM nifedipine, to block CaV1.x, and 0.1 mM Ni^{+} to block CaV3.X, together with standard Na^{+} and K^{+} channel blockers and n-methyl-D glucamine^{+} as the primary cation, we noted two single inward channel conductances: ~1.4 pS and ~2.5 pS (*N=4*). The larger channel was identified as CaV2.2 since it was absent in 4 of 4 patches with ω-conotoxin GIVA (2.5 μM), a specific CaV2.2 blocker, but was present in 7 out of 8 patches with 2 mM $[Ca^{2+}{}_{ext}]$ or $[Ba^{2+}{}_{ext}]$ whereas the small channel remained (*N=3*). Thus, our data indicate that at physiological $[Ca^{2+}]_{ext}$, CaV2.2 has a much higher conductance, and hence larger single channel domain, than predicted.

1146-Plat
Gating Charge Movement Is Prevented By Open State Occupancy Of N-type (CaV2.2) Calcium Channels
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L-type calcium channels show a loose coupling between channel closing and gating charge movement. There are significant gating differences between N-type and L-type channels and we wondered if some of these differences were linked to the relationship between charge movement and channel opening. This was accomplished by comparing the time constant (τ) for channel closing (τ_{Deact}) with that for Off-gating charge movement (τ Q_{Off}) over a range of voltages. Ionic currents were recorded in 5 mM Ca^{2+}, while gating currents were recorded in 0.1 mM La^{3+} and 5 mM Mg^{2+} (La-Mg) from N-channels expressed in HEK 293 cells. τ Q_{Off} was larger than τ_{Deact} and the voltage dependence of the τ Q_{Off} was less steep than that for τ_{Deact}, which suggests that gating charge relaxation does not limit channel closing. To determine if the reverse was true, we used roscovitine, which slows N-channel closing by holding the channel in a high P_o open state. We found that τ Q_{Off} was identical to τ_{Deact} in roscovitine. There was a risk that residual ionic tail current could contaminate Off-gating current, so we used an envelope protocol to measured the recovery time course of Q_{On} (no ionic current contamination), and found the same τ as for both τ Q_{Off} and τ_{Deact} in roscovitine. This coincidence of τ Q_{Off} with τ_{Deact} suggests that transition out of the roscovitine-bound high Po open state becomes rate limiting to both Q_{Off} and channel closing. We conclude that, unlike L-channels, the high P_o N-channel open state places the channel into a confirmation that locks gating charge into the activated position.

1147-Plat
Cardiac Alpha$_{1a}$-adrenoceptor Stimulation Inhibits L-type Ca^{2+} Current In The Presence Of Beta-adrenoceptor Stimulation Through Tyrosine Kinase
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Introduction: We previously showed that cardiac α_1-adrenoceptor (AR) stimulation alone potentiates L-type Ca^{2+} current (I_{Ca}) through α_{1A}-AR-PLC-PKC pathway (O-Uchi J et al. ***PNAS.***, 2005 and ***Circ Res.***, 2008). However, the interaction of α_1- and β-AR signalings for I_{Ca} regulation was not fully clarified. In the present study, we examined the effect of α_1-AR stimulation on I_{Ca} when β-AR is stimulated. **Methods**: Perforated patch-clamp was used for recording I_{Ca} from isolated adult rat ventricular myocytes. Cells were at first treated with β-AR agonist (100 nM isoproterenol) for 5 min and then α_1-AR agonist (100 μM phenylephrine) was applied in the continuous presence of isoproterenol. Holding potential was set at -40 mV and depolarization pulse to 0 mV was applied every 10 sec. **Results**: Phenylephrine significantly inhibited I_{Ca} in the presence of isoproterenol by 19.6±7.6%. The α_{1A}-AR selective antagonist (WB4101) blocked this inhibitory effect by phenylephrine, but α_{1B}-AR selective antagonist (L-765,314) did not, confirming that only α_{1A}-AR is involved in this inhibitory effect. Phenylephrine had no effect on I_{Ca} activated by forskolin. In addition, inhibition of G_q signaling by PLC inhibitor (U73122) or inhibition of $G_{i/o}$ signaling by pertussis toxin did not blocked the phenylephrine-induced inhibition of I_{Ca}. The tyrosine kinase inhibitor (lavendustin A) attenuated the response of phenylephrine during β-AR stimulation. **Conclusion**: α_{1A}-AR stimulation inhibits I_{Ca} in the presence of β-AR stimulation, which is opposite to the effect observed in the absence of β-AR stimulation. This effect is not mediated through G_q and $G_{i/o}$ but through tyrosine kinase activity, which inhibits the upstream of β-AR signaling (at the level of β-AR or Gs). The inhibitory effect of α_{1A}-AR stimulation could serve as one of the regulatory feedback mechanisms when catecholamine level increases under pathophysiological conditions.

1148-Plat
Calmodulin Regulates Calcium Sparklet Activity in Vascular Smooth Muscle
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Calcium influx through L-type calcium channels (LTCCs) influences numerous physiological processes in excitable cells ranging from contraction, memory and gene expression. Clusters of LTCCs can operate in a PKCalpha-dependent, high open probability mode that generates sites of sustained calcium influx called "persistent calcium sparklets". In vascular smooth muscle, persistent calcium sparklets contribute to local and global calcium. Calcium sparklets activity varies regionally within smooth muscle cells. At present however, the mechanisms underlying heterogeneous sparklet activity are incompletely understood. Here, we use TIRF microscopy and whole-cell patch clamp electrophysiology to investigate the role calmodulin in the modulation of calcium sparklet activity. We found that inhibition of calmodulin increases calcium sparklet activity in wild type (WT) smooth muscle cells. Inhibition of calmodulin in PKCalpha KO cells, which are devoid of persistent calcium sparklets, increased calcium influx by evoking new persistent calcium sparklet sites and by increasing the activity of previously low activity sites in these cells. On the basis of these finding, we hypothesize that calmodulin plays a critical role in determining the activity of calcium sparklet sites in arterial smooth

muscle. This work was supported by the National Institutes of Health and the American Heart Association.

Platform Z: Cardiac Muscle I

1149-Plat

Ventricular Myocyte Morphology in Long Term Culture

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Previously we have introduced a single cell system allowing long term culturing (1 week) of adult rat ventricular myocytes while maintaining their overall morphology, contractile behaviour and calcium-signalling.

Here, we characterize the subcellular morphology of the myocytes, including the Golgi apparatus, endo-/sarcoplasmic reticulum (ER/SR), plasma membrane and mitochondria. Cells were isolated from adult rats following a standard enzymatic procedure.

Organelles were labelled using targeted expression of fluorescent proteins, e.g. dsRed1 fused to the subunit VIII of human cytochrome C oxidase for mitochondria, YFP fused to a GPI-anchor for the plasma membrane, YFP fused to ts045G for the Golgi apparatus and dsRed2 fused to calreticulin for the ER/SR. Complementing this we also applied fluorescent dyes; di-8-ANEPPS for the plasma membrane and MitoTracker Green for the mitochondria. 3-dimensional stacks of individual cells were acquired with a nipkow-disc based confocal microscope.

Using both labelling approaches, the analysis of the plasma membrane illustrated a gradual loss of the t-tubules during culturing with cytosolic membrane fragments being present for extended time periods.

Mitochondria, which are very prominent and densely packed in cardiac myocytes, underwent an apparent fusion of originally isolated mitochondria, possibly reflecting the loss of t-tubules. While the structure of the ER/SR remained unaltered, the Golgi apparatus underwent a significant redistribution during the culturing time from a wide cytosolic distribution to a perinuclear accumulation.

We provide important evidence that cell morphology changes unavoidably occurring in adult cardiac myocytes during long term culture are highly reproducible and thus strongly support the application of such a single cell model in high-content screening applications.

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1150-Plat

Calcium Independent Positive Inotropy By Fast Cardiac Myosin Motor Gene Transfer In Slow Myosin Dominant Ventricular Myocytes

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Current inotropic drug therapies used to boost cardiac muscle performance focus on elevating the amount of calcium that is mobilized for activation of the myofilaments. These calcium based therapies can provide short term benefits, but when administered long term can actually increase mortality due to calcium overload and the development of fatal arrhythmias. Calcium mobilization has also been the key target for current gene therapy strategies (i.e. SERCA2a over-expression, PLN knockdown, S100 protein expression) to treat the failing heart. Here we present a novel form of calcium independent positive inotropy by fast cardiac myosin motor gene transfer. We designed a recombinant adenovirus to express the full length human α-myosin heavy chain (α-MyHC, *MYH6*) gene in rabbit or human ventricular myocytes that endogenously express almost exclusively β-MyHC. Healthy or diseased adult cardiac myocytes were isolated by enzymatic digestion and maintained in primary culture for 48 hours. Highly efficient α-MyHC gene transfer was confirmed by fluorescent immunocytochemistry and Western blotting. In all cases contractility of single cardiac myocytes was measured 48 hours after gene transfer of α-MyHC by measuring sarcomere shortening and intracellular calcium transients. Sarcomere shortening was ~35% greater in cardiac myocytes transduced with the α-MyHC adenovirus when α-MyHC made up ~30% of the total myosin protein. Intracellular calcium transient amplitudes, however, were not affected by α-MyHC gene transfer. In permeabilized myocyte experiments we found that α-MyHC gene transfer did not affect myofilament calcium sensitivity, but did speed the kinetics of myosin cross bridge transitions from weakly-bound to strongly-bound states. We conclude that α-MyHC gene transfer offers a novel form of calcium independent positive inotropy for β-MyHC dominant adult ventricular myocytes.

1151-Plat

Role of Thin Filament Cooperative Activation in Length-dependent Activation in Skinned Porcine Ventricular Muscle

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The basis of the Frank-Starling mechanism of the heart is the intrinsic ability of cardiac sarcomeres to produce greater active force in response to stretch (i.e., length-dependent activation). We have reported that troponin plays a key role in this phenomenon via on-off switching of the thin filament state, in concert with titin-based passive force (Terui et al., *J Gen. Physiol.* 131;275-283:2008). In the present study, we systematically investigated the role of thin filament cooperative activation in length-dependent activation using skinned porcine ventricular muscle at sarcomere lengths of 1.9 and 2.3 μm. MgADP (3 mM) increased Ca^{2+} sensitivity of force and enhanced the speed of contraction, indicating enhanced cooperative activation. MgADP was found to attenuate length-dependent activation, with and without quasi-complete reconstitution of thin filaments with fast skeletal troponin (sTn; from rabbits). Conversely, inorganic phosphate (Pi, 20 mM) decreased Ca^{2+} sensitivity of force and the speed of contraction, indicating reduced cooperative activation. Pi enhanced length-dependent activation, with and without sTn reconstitution. Qualitatively similar results were obtained with MgADP or Pi in rabbit fast skeletal muscle, with higher Ca^{2+} sensitivity of force than in cardiac muscle. Linear regression analysis revealed that the speed of contraction, Ca^{2+} sensitivity of force and length-dependent activation were strongly correlated in both cardiac and skeletal muscle. These results suggest that length-dependent activation is regulated via thin filament cooperative activation, such that the length-dependent increase in the fraction of cross-bridges is less in high cooperative activation states, coupled with a loss of recruitable cross-bridges.

1152-Plat

Length-Dependent Active Tension Development In Single Intact Cardiomyocytes, Isolated From Different Regions Of Guinea Pig Heart

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Introduction: Information on the force-length relation of intact myocytes from different regions of the heart is scarce. We therefore studied myocytes, isolated from apical and basal areas of guinea pig left and right ventricles (cell numbers: LV_A 22, LV_B 29, RV_A 11, RV_B 12). **Methods:** Force-length relations were measured by attaching carbon fibers to myocytes, allowing application of diastolic stretch while measuring passive and active force.[1] Cells were kept at 36 ± 1°C and paced at 2 Hz. Recorded forces were normalized to cell cross-sectional area and used to construct end-diastolic, end-systolic and active tension-length relations (EDTL, ESTL and ATL=ESTL-EDTL; respectively). In addition, the ratio of the slopes of ESTL and EDTL was used as a cross-section independent factor to characterise the Frank-Starling Gain (FSG) in individual cells. **Results:** For all tissue regions ESTL, EDTL and, hence, ATL, are linear over the range of end-diastolic sarcomere lengths studied (1.88-2.15 μm). Plotting the slope values of ATL vs. EDTL for all cells shows a positive correlation (slope 1.29, R^2=0.24, 74 cells). In addition, FSG is larger than one for all cells studied: RV_B 3.91 ± 0.54), RV_A (2.84 ± 0.30), LV_B (2.77 ± 0.17), and LV_A (3.20 ± 0.24). **Conclusions:** Using the carbon fiber technique, it is possible to probe length-dependence of passive and active tension at the single cell level, without the interference of extracellular structures. The Frank-Starling Gain varies between the different regions of the heart and the positive correlation between ATL and EDTL confirms that in intact cells passive force bearing structures (such as titin) are likely to play a role in modulating length dependent activation.

[1] Iribe et al, Force-length relations in isolated intact cardiomyocytes subjected to dynamic changes in mechanical load. AJP 2007/292:1487-1497.

1153-Plat

Sarcomere Length Dependence Of The Force-pCa Relationship In Cardiac Muscle Is Influenced By Properties Of Thin Filament Regulatory Units

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Myocardial performance is tightly regulated by sarcomere length (SL), which is thought to be due at least in part to concurrent changes in myofilament lattice spacing and the relative proximity (and binding probability) of myosin heads to actin. Furthermore, crossbridge binding enhances Ca^{2+} binding to troponin (Tn) in cardiac muscle, a unique form of cooperative thin filament activation