a high-speed complementary metal oxide semiconductor camera to track the movements of hundreds of cells in parallel from dozens of preparations. This work revealed that the spontaneous oscillations exhibit multiple timescales with a slow modulation on a rapid oscillation. Experiments inhibiting the electrical resonance in the cell body show a strong effect on the mechanical oscillations of the hair bundles. This indicates that the electrical oscillation is coupled with the mechanical oscillations of the hair bundles.

2636-Pos

Effect of Having Two Identical Channels Per Tip-Link in Hair Bundles

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Gating of mechanoelectrical transducer (MET) channels in hair bundles of hair cells is the key step of mechanosensitivity, including sensing sound and balance. Transducer current, gating compliance, which can lead to negative stiffness of the hair bundle, and adaptation have been successfully described by theoretical models that assume that a single MET channel is connected to each tip-link.

However, recent experimental reports have demonstrated that each tip-link is associated with more than one channel. This discrepancy begs an answer to the question: What are the consequences of two MET channels being associated with a single tip-link?

To address this question, we assume that two channels associated with each tip-link are identical and examine the effect of two basic connectivities, series and parallel, of the channels on their gating. We found that the connectivity has much larger effect on gating compliance than on transducer current. With series connection, gating compliance has a single maximum only if it is below a critical value. Further increase of gating compliance splits its maximum into two. Thus negative stiffness, the result of a further increase in gating compliance, appears in two regions on the displacement axis. In contrast, parallel connection leads to gating compliance similar to that predicted by the previous model that assumes a single channel is associated with each tip-link. Although some cooperative effect of the two channels is present, it cannot be significant for sensitive hair bundles. For those reasons, parallel connection of two identical channels is consistent with experimental observations.

2637-Pos

Structural Determinants of Cadherin-23 Function in Hearing and Deafness

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The tip link is an essential component of the hair-cell transduction apparatus and has been proposed to be part of a biophysically defined "gating spring". Cadherin-23 and protocadherin-15 likely form the tip link; both proteins belong to the cadherin superfamily of proteins that mediate calcium dependent cell-cell adhesion and are involved in hereditary deafness, yet their molecular structures and elasticity are unknown. Here we present crystal structures for cadherin-23 repeats 1 and 2 (EC1+2). Overall, the structures show a typical cadherin fold for both repeats, but reveal an elongated N-terminus that impairs classical cadherin-cadherin interactions and facilitates the formation of an additional calcium binding site. The linker region between the repeats closely resembles a classical cadherin calcium-binding motif and contains the site of the D124G mutation causing non-syndromic deafness (DFNB12). The crystal structure of EC1+2 carrying this mutation displays a different angle between the repeats than the wild-type protein. Molecular dynamics simulations of wild type structures and various mutant models suggest that deafness mutations and removal of calcium ions control cadherin inter-repeat motion and unfolding strength of hair-cell tip links. The structures along with simulations indicate that cadherin repeats forming the tip link are too stiff to be the gating spring. In addition, the new structures define a previously uncharacterized family of cadherin proteins and begin to suggest mechanisms underlying disease as well as ways in which cadherin-23 may bind end-to-end with itself and also with protocadherin-15 to form the tip link.

Ryanodine Receptors II

2638-Pos

Emerging of Ryanodin Receptors Mediated Calium Signaling Synchroonizes Early Cardiac Contractility

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Stem cell transplantation is a promising technique to improve cardiac function. However arrhythmias in regenerating areas are a major concern surrounding the use of cellular cardiomyoplasty. We associate these arrhythmias with developmental changes in intracellular calcium (Ca^{2+}) signaling. Cellular contractility in early cardiac development induced by Ca^{2+} oscillations evoked by Ca^{2+} release from Inositol 1,4,5-triphosphate receptors (IP3Rs), whereas in adult myocytes Ca^{2+} released from the ryanodine receptors (RyR2s) is the main source of cytosolic Ca^{2+} elevation. Recently we have shown that cardiac specific RyR2s mediated Ca^{2+} signaling in early cardiogeneses does exist in early cardiogenesis but functionally silent due to the lack of sufficient luminal Ca^{2+} reserve. Adenovirus mediated calsequestrin (CASQ2) gene transfer in mouse embryonic stem cells potentiated expression of CASQ2 protein in early cardiac development and contributed to the emergence of RyR2s mediated Ca^{2+} signaling in the developing cardiac myocytes. Expression of CASQ2 significantly increased synchronization contractility of early stage (7+1days after in vitro induction of cardiac differentiation from mouse embryonic stem cells cardiac myocytes) in a single cluster of contracted cells (94% of clusters contracted synchronously vs 14% in control). Depletion of RyR2s associated Ca^{2+} stores with caffeine demolished synchronization and reduced frequency of cellular contractility (31 ± 3 vs 48 ± 5 bpm in control). Similar changes were observed in 7+7 differentiation stage cardiomyocytes differentiated from wild type ES cells. Caffeine reduced number of synchronously contracted clusters from 84% to 28%. Frequency of cellular contractility was also reduced from 46 ± 3 to 34 ± 4 bpm.

We conclude that establishment of RyR2s mediated Ca^{2+} signaling in early cardiogenesis is important for synchronization of early cardiac contractility. Ectopic expression of CASQ2 in early cardiogenesis leads to creation of functional RyR2 Ca^{2+} stores and has major impact on stabilization of intercellular contractility.

2639-Pos

Calcium Influx Analysis by TIRF Microscopy on Cultured Primary Myotubes from Patients with *RyR1* Mutations

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Central core disease (CCD) and malignant hyperthermia (MH) have been linked to point mutations in the gene encoding the skeletal muscle sarcoplasmic reticulum calcium release channel (ryanodine receptor), which is localized on human chromosome 19 (*RYR11*). Central core disease is a relatively mild, slowly progressive autosomal dominant myopathy, characterized histologically by the presence of centrally located cores running the length of the muscle fibres. MH is a pharmacogenetic induced hypermetabolic disease. CCD linked RyR1 mutations are associated with depletion of thapsigarin-sensitive stores and to an increase of the resting calcium level. Influx of Ca^{2+} from the extracellular environment is a major factor which influences the level of resting cellular [Ca^{2+}]. Our working hypothesis is that decrease of sarco(endo)plasmic reticulum Ca^{2+} load via leaky ryanodine receptor channels and/or alteration of calcium influx via store operated channels or excitation-coupled Ca^{2+} entry (ECCE), may account for, at least in part, the phenotype of patients with CCD, including muscle weakness and abnormal secretion of inflammatory cytokines from muscle cells and cells of the immune system. We set out to test the validity of our hypothesis by directly investigating the mechanisms activating calcium influx in myotubes from normal individuals and from patients with CCD and MH by TIRF microscopy. Our data shows that mutations in the RyR1 affect ECCE in human myotubes from CCD and MH patients. *This work as supported by grants from A.F.M., S.N.F., Telethon Italy*

2640-Pos

Dysregulation of Ca^{2+} Entry and SR Calcium Leak are Responsible for Elevated Resting Free Ca^{2+} in Triadin-Null Myotubes

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Previously we have shown that lack of triadins expression in skeletal muscle cells results in significant increase of myoplasmic resting free Ca^{2+} ($[Ca^{2+}]_r$), suggesting a role for triadins in modulating global intracellular Ca^{2+} homeostasis. To further characterize this mechanism, here we study the effect of triadin on Ca^{2+} entry, $[Ca^{2+}]_r$, and Ca^{2+} release pathways using Mn^{2+} quench, Ca^{2+} selective microelectrodes and Ca^{2+} imaging in Wt and triadin-null myotubes. Comparison of Mn^{2+} quench rates in resting conditions revealed that triadin-null cells have higher Ca^{2+} entry rates than Wt cells. However, prevention of Ca^{2+} entry with Cd^{2+} and La^{3+} was insufficient to restore

$[Ca^{2+}]_r$ of triadin-null cells to levels similar to Wt cells, suggesting either an additional Ca^{2+} entry pathway, insensitive Cd^{2+} and La^{3+}, or an enhanced Ca^{2+} release. Inhibition of Ca^{2+} release by ryanodine resulted in a significant reduction of $[Ca^{2+}]_r$ in triadin-null cells but not in Wt, indicating that triadin-null cell also have increased baseline RyR1 channel activity. Western blot analysis and lipid bilayer studies revealed that calcium channels from triadin-null cells have reduced FKBP-12 binding and increased subconductance states, respectively. Accordingly, over expression of FKBP-12.6 caused a significant reduction in $[Ca^{2+}]_r$ in triadin-nulls but did not affect Wt cells. Overall these data support the idea that elevated resting free calcium levels observed in triadin-null myotubes are the combined effect of at least two things: (i) an increase in calcium entry mediated by Cd^{2+} and La^{3+} sensitive channels, but insensitive to nifedipine, and (ii) an augmented basal SR calcium release as the result of enhanced RyR1 channel activity induced by a deficiency in RyR1/FKBP-12 binding.

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2641-Pos

The Leak State of the RyR1 is Regulated by RyR1/DHPR Interaction, Controlling the Cytosolic Free-Ca^{2+} Concentration and the SR Ca^{2+} Content at Rest

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The sarcolemmal L-type Ca^{2+} channel (DHPR), is known to both positively and negatively regulate RyR1 in skeletal muscle. The mechanism for the negative control is unknown. To assess this we measured resting intracellular Ca^{2+} concentration ($[Ca^{2+}]_{rest}$) using Ca^{2+}-selective microelectrodes in dysgenic (MDG) and Wt myotubes. The $[Ca^{2+}]_{rest}$ in MDG and Wt myotubes were 158 ± 2.5 nM (mean ± SE, n = 9) and 117 ± 2.1 nM (n = 10), respectively ($p<0.001$). Bastadin 5 (B5), which suppresses the leak state of RyR1, decreased $[Ca^{2+}]_{rest}$ to 99 ± 0.7 nM (n=16) and 103 ± 1.3 nM (n=16) in Wt and MDG myotubes, respectively. After FK506 treatment, $[Ca^{2+}]_{rest}$ increased to 139 ± 2.4 nM (n=10), 217 ± 3.0 nM (n=10) in Wt and MDG myotubes, respectively and the addition of B5 in FK506 treated cells had negligible effect: 129 ± 1.8 nM (n=10) and 202 ± 3.5 nM (n=10) respectively. These experiments clearly show that B5 treatment equalizes the $[Ca^{2+}]_{rest}$ in all the three cell types tested while FK506 abolishes its action. To estimate the SR Ca^{2+} content, we measured Ca^{2+} release elicited by 20 mM caffeine, using Fluo5N. The area under the curve of the Fluo5N signal was ~4 times smaller in MDG (25 ± 2.3 arbitrary units (a.u.), n=69) compared to Wt (107 ± 8.5 a.u, n=66) and B5 increased the Ca^{2+} release only in MDG cells: 76 ± 16 a.u. (n=21) MDG vs 101 ± 12 a.u. (n=60) Wt. These data demonstrate that the negative control exerted by the DHPR is at least in part due to the ability of the DHPR to control the percent of RyR1s in the leak state. Supported by NIH/NIAMS R01AR43140 (to PDA and INP)

2642-Pos

Increased Sensitivity of RyR2 to Activation by Ca^{2+} and cADP-Ribose during Diabetes

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In prior studies others and we found an increase in spontaneous Ca^{2+} release in ventricular myocytes isolated from streptozotocin-induced diabetic rat hearts. To date, molecular mechanisms underlying this phenomenon remains poorly defined. This study tests the hypothesis that the sensitivity of RyR2 to be activated by intrinsic modulators is altered during diabetes. Diabetes was induced in male Sprague-Dawley rats using streptozotocin. Ten weeks after injection, diabetic rats were divided into two groups: one group was treated with insulin for two weeks while the other group received no treatment. At the end of this time, cardiac and myocyte functions were assessed using echocardiography and high-speed video edge detection. Evoked Ca^{2+} transients were also assessed using confocal microscopy. Expression of RyR2 was assessed using RT-PCR and Western blots, while RyR2 activity and responsiveness to Ca^{2+}, cADP-ribose and AMP-PCP were assessed using [^{3}H]ryanodine binding and single channel analyses. After 12 weeks of diabetes, ejection fraction and fraction shortening were reduced by >25%. Rate of evoked Ca^{2+} release was slowed as was the time to peak myocyte contraction. Expression of RyR2 and the total amount of [^{3}H]ryanodine bound at 200μM Ca^{2+} were reduced by ≈ 40%. However, the K_d for ryanodine remained essentially unchanged (5.2nM). Interestingly, in [^{3}H]ryanodine binding and single channel assays, diabetic RyR2 was activated to a greater extentby low Ca^{2+} (0.53 μM and 1 μM), AMP-PCP (1mM) and cADP-ribose (1 μM). Two-weeks of insulin, initiated after 10 weeks of diabetes, treatment blunted these changes. These new data indicate that the increase in spontaneous Ca^{2+} release seen in diabetic myocytes stems in part from alterations in the responsiveness of RyR2 to activation by intrinsic ligands. Funded in part by NIH HL085061 and the Nebraska Redox Biology Center.

2643-Pos

Effects of Divalent Current Carriers on Voltage-Dependence of RyR2 Channels

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Voltage-dependent modulation of cardiac ryanodine receptors (RyR2) was studied in planar lipid bilayers. Different earth alkaline cations (M^{2+}: Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}) were utilized as current carriers. When added to the cytosolic surface of the channels, Ca^{2+} (EC_{50} ~2μM) or Sr^{2+} (EC_{50} ~20μM) interacted with cytosolic high affinity (HA) sites and activated the channels. Neither Mg^{2+} nor Ba^{2+} activated RyR2 but only Mg^{2+} interfered with Ca^{2+}/Sr^{2+} action. Fully activated RyR2 (100-200μM cytosolic Ca^{2+}) were inhibited with low affinity (LA) by all M^{2+} (similar IC_{50}, ranging 3.6-5.7mM) suggesting that LA inhibitory sites do not distinguish M^{2+} identity. We found that RyR2 were much more active with lumenal Ca^{2+}/Sr^{2+} versus lumenal Ba^{2+}/Mg^{2+}. However increasing lumenal→cytosolic M^{2+} flux by increasing lumenal holding voltage (Vm) never increased open probability (Po). In contrast, increasing Vm decreased Po in half of the RyR2 exposed to lumenal Ca^{2+}/Sr^{2+} and in all RyR2 exposed to lumenal Ba^{2+}/Mg^2. This suggests that lumenal flux does not reach HA but LA sites. An effect of Vm is evident in all channels displaying modal gating (low and high Po mode) but not in RyR2 where high Po dominates. Indeed, high Po mode is much more abundant with lumenal Ca^{2+}/Sr^{2+}. Moreover, increasing cytosolic Ca^{2+} activated all channels and removed the effect of Vm. In summary, modulation of RyR2 gating by M^{2+} flux is complex and seems to reflect lumenal M^{2+}-dependent stabilization of high Po and low Po mode (the latter being voltage-dependent). Supported by NIH R01 GM078665 to JAC.

2644-Pos

Molecular Cloning and Expression of the Ryanodine Receptor Type 2 (RyR2) from Rat Cerebral Artery Smooth Muscle

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Ryanodine receptors (RyRs) are a family of Ca^{2+} release channels found in intracellular Ca^{2+} storage/release organelles and participate in a variety of important Ca^{2+} signaling phenomena. Here, we set to clone and sequence full-length cDNA encoding the type 2 RyR (RyR2) from smooth muscle cells of *Rattus norvegicus* cerebral arteries. Middle cerebral and basilar arteries were isolated and de-endothelized, and total RNA was purified for RT-PCR. RyR2 cDNA was divided in two parts of similar size; 5' segment: 1-7486 bp, and 3' segment: 7487-14862 bp. Specific primers were designed to obtain both 5' and 3'-terminals. Following insertion of both terminals in the mammalian expression vector pCI-neo, we characterized several clones by restriction analysis, and confirmed the full-length cDNA sequence by automated sequencing. The rat cerebral artery smooth muscle RyR2 cDNA contains 14862 bp and encodes a deduced protein of 4953 amino acids with a M_r of 562451.3 Da. Nucleotide blast analysis indicates that the cerebral artery smooth muscle RyR2 shows 100% identity with recombinant RyR2 cloned from rat cardiac muscle (Accession NM_032078). RyR2 cDNA expression was determined after transfection of HEK293 cells with the insert into the pCMV6-AN-tagGFP vector. Membrane insertion of cerebral artery myocyte RyR2 was determined by immunolabeling with polyclonal antibodies. To our knowledge, this is the first time that a RyR is cloned from rat cerebral artery smooth muscle, its functional characteristics being currently studied. *Supported by Grant AA11560 (AMD).*

2645-Pos

Bacterial Expression of the Ryanodine Receptor Pore Forming Region and a Potassium Channel Chimera

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Several lines of evidence exist to support the proposal that RyR2 contains a pore composed of structural elements analogous to the pore forming regions (PFR) of K+ channels. Our analogy model, constructed using the bacterial