determine the fundamental mechanism for CICR activation in mammalian skeletal muscle. Transient osmotic stress increases PI(4,5)P2 and inositol 1,4,5 triphosphate (IP3) levels. Application of wortmannin or xestopongin C significantly reduces osmotic stress-induced Ca^{2+} spark activity in intact muscle fibers, suggesting the role of IP3 receptor in Ca^{2+} spark signaling. Western blot shows that both IP3 receptor type 1 and 2 are present in adult skeletal muscle, and immunostaining reveals that both IP3 receptors are distributed along the sub-sarcolemmal region of the muscle fiber (with some concentrated to the perinuclear area). Using electroporation mediated transfection to deliver short hairpin (sh)RNA that targets IP3 receptors, we are able to knockdown the expression of both IP3 receptors 1 and 2 in the muscle of viable adult mice. We find that reduced expression of IP3 receptors ablates osmotic stress-induced Ca^{2+} spark activity, indicating Ca^{2+} sparks activity in skeletal muscle requires activation of IP3 receptor. Thus, osmotic stress-induced Ca^{2+} spark signaling in skeletal muscle requires two cellular events: first, uncoupling of the inhibitory role of the voltage sensor on the RyR1 channels, and second, production of the IP3 second messenger near the sarcolemmal membrane. These results represent the first description of IP3 receptors producing CICR from RyR1 in mammalian skeletal muscle and provide essential clues to the function of these Ca^{2+} sparks in skeletal muscle physiology.

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Recovery of the Compromised Ca^{2+} Spark Signaling in Aged Skeletal Muscle Through Restoration of MG29

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Sarcopenia is a degenerative loss of skeletal muscle function associated with aging. Our previous results identify reduced MG29 expression in aged skeletal muscle, and mirroring phenotypes of the young MG29 knockout and aged wild type muscles in that both show reduced Ca^{2+} spark response to osmotic-stress. Thus, compromised intracellular Ca^{2+} homeostasis due to reduced MG29 expression may be one of the underling mechanisms for aging-related skeletal muscle dysfunction. Here we explored the effects of MG29 rescue on Ca^{2+} spark signaling in aged skeletal muscle. Electroporation-based method was used to introduce MG29 into *flexor digitorum brevis* (FDB) muscle and adeno-associated virus (AAV)-based method was used to deliver MG29 gene into the hindlimb of the living mice. Confocal microscopic imaging revealed increased Ca^{2+} spark events in aged FDB muscle following transient overexpression of MG29. These Ca^{2+} sparks showed plastic response to osmotic stresses, similar to those observed in the young wild type muscle. 2-3 weeks following AAV-mediated delivery of MG29, the aged skeletal muscle showed only marginal increase in contractile force as compared to the contralateral controls. Our data suggest that transient restoration of MG29 expression in aged muscle has beneficial effects on improvement of intracellular Ca^{2+} signaling. Since MG29 is involved in maintenance of the transverse-tubule network, restoration of contractile force in aged muscle may require sustained elevation of MG29 to allow for remodeling of the disrupted membrane network.

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Hypersensitive Intracellular Ca^{2+} Signaling Precedes Deterioration of Cardiac Functions in Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a severe form of striated muscle disease. Although respiratory failure remains a leading cause of death, a number of patients succumbs from cardiac manifestations of the disease. The *mdx* mouse, an animal model of DMD, develops progressive dilated cardiomyopathy. Several studies associated changes in Ca^{2+} homeostasis with the disease. Here we investigated whether these changes were causal for or a consequence of the pathology. Ca^{2+} handling was studied in intact and patch-clamped cardiomyocytes isolated from 1 to 4 month old mice. According to several reports, young *mdx* mice show no significant changes in cardiac performance. However, even myocytes from 1 month old *mdx* mice produced exaggerated Ca^{2+} signals in response to osmotic shock, and exhibited "hypersensitive" excitation-contraction coupling (ECC gain was more resistant to a reduction in $[Ca^{2+}]_{ex}$ in *mdx* than in WT cells). Ca^{2+} transients induced by osmotic shock were nearly abolished by the super-oxide dismutase mimetic Mn-cpx3, substantially reduced by a CaMKII inhibitor (KN-93) and partially diminished by PKA inhibitors (KT5720, H89). No significant changes in SR Ca^{2+} load as well as in resting $[Ca^{2+}]_i$ were found in young *mdx* compared to WT cells. Together with our previous results, these data suggest that 1) increased sensitivity of RyRs to Ca^{2+} precedes and probably contributes to the development of cardiomyopathy in dystrophy and that 2) there is a synergistic interaction among several pathomechanisms which hypersensitize the RyR. This includes a) abnormal Ca^{2+} influx resulting in b) cellular Ca^{2+} overload, c) elevated ROS generation leading to RyR redox modification and sensitization, and d) activation of protein kinases with subsequent RyR phosphorylation and even further sensitization. Thus, future pharmacological strategies should preferably target several of these mechanisms contributing to abnormal Ca^{2+} signals in DMD.

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A Novel Role for Polyphosphate in Astrocyte Signalling

Kira M. Holmstrom, Alexander V. Gourine, Andrey Y. Abramov.

Inorganic polyphosphate exists in nature in varying lengths from tens to thousands of orthophosphates linked by high energy bonds similar to ATP. The polymer is highly conserved from bacteria to human, but although its role has been extensively studied in bacteria, its function in the mammalian cell is only slowly coming to light. Polyphosphate has been detected in the rodent brain at micromolar concentrations and has been shown to regulate ion channels in neurons, suggesting that polyphosphate may play a role in neuronal signalling.

We used fluorescent live cell imaging to investigate the response to polyphosphate in primary astrocytic and neuronal co-cultures. For the experiments three different lengths of polyphosphate (short −14, medium −60, and long −130, orthophosphates) were used. Further, using the ratiometric Ca^{2+} indicator fura-2, we were able to identify a transient Ca^{2+} signal, mainly in astrocytes, in response to polyphosphate in the range of 10-100μM for all three lengths of the polymer. Interestingly, inhibiting phospholipase C by U73122 abolished the Ca^{2+} transient, as did emptying the endoplasmic reticulum of Ca^{2+} before addition of polyphosphate, using the sarco/endoplasmic reticulum Ca^{2+} ATPase inhibitor thapsigargin. On the other hand, removal of Ca^{2+} from the extracellular recording medium did not alter the signal, suggesting that the Ca^{2+} signal stems from the endoplasmic reticulum and is mediated through phospholipase C and IP_3 activation. Further characterisation, using different cell surface receptor inhibitors, suggests that the signal is mediated through purinergic receptors, as the broad spectrum P2 inhibitors PPADS and suramin both block the signal.

These novel findings highlight the possible importance of polyphosphate in signal transmission in the brain.

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Dynamic Control of Neuronal Firing Threshold by Calcium Buffering: A New Role for Calcium Binding Proteins

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We have investigated the detailed regulation of neuronal firing threshold by the cytosolic calcium buffering capacity using a combination of mathematical modeling and patch clamp recording in acute slice. Theoretical results show that, at similar free calcium concentration, increased calcium buffer concentration lowers the firing threshold of cerebellar granule cells. We show that this effect is a direct consequence of the major slowdown of calcium dynamics. Patch clamp recordings on cerebellar granule cells loaded with a high concentration of the fast calcium buffer BAPTA (15 mM) reveal alterations in the excitability threshold as compared to cells loaded with 0.15 mM BAPTA. In high calcium buffering conditions, granule cells exhibit a significative lower firing threshold. These results suggest that cytosolic calcium buffering capacity can tightly modulate neuronal firing threshold and therefore that calcium-binding proteins may play a critical role in the information processing in the central nervous system.

Intercellular Communications & Gap Junctions

3054-Pos Board B159

Single Hemichannels Recorded in Lipid Bilayers and Artificial Gap Junction Formation with Cells

Mohamed Kreir, Christoph Methfessel, Christian Carnarius, Claudia Steinem, Niels Fertig.

Connexins (Cx) are members of a multigene family of membrane-spanning proteins that form gap junctions, which are composed of two hexameric hemichannels, called connexons. These gap junctions, organized in so-called gap junctional plaques, span the extracellular space/matrix of adjacent cells and thus allow a passive exchange of small molecules up to about 1 kDa. Connexins are widely distributed with various subtypes of connexin and are involved in different biological processes such transmission of information and propagation of action potential for e.g. Recent studies indicates that hemichannels do open under physiological and pathological conditions.

In our study, we investigated the biophysical properties of hemichannels Cx26 and Cx43 which were isolated biochemically and reconstituted into synthetic lipid membranes. Both hemichannels are present in different tissues and involved in different pathologies. The results on a study of the Cx26 are presented. Reconstitutions of functional Cx26 and mutant hemichannels were performed. Secondly, Cx43 was purified and reconstituted into bilayers. The hemichannel Cx43 properties were compared to previous studies and showed similarities of conductance on single channel recordings of Cx43 in cells. Our focus was then to form artificial gap junctions, first between two unrelated cells and then between cells and bilayers containing functional hemichannels. This was done using Cx26 or Cx43. The bilayer-cell configuration allows to measure electrophysiological properties of the cells indirectly via gap junctions. Single channel recordings of gap junctions were recorded using a bilayer containing Cx43 and Cardiomyocytes expressing Cx43. Macroscopic currents were as well recorded between bilayers and cell lines expressing Cx26 or Cx43.

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Asparagine175 of Cx32 is a Critical Residue for Docking and Forming Functional Heterotypic Gap Junction Channels with Cx26

So Nakagawa, **Xiang-Qun Gong**, Shoji Maeda, Yuhua Dong, Yuko Misumi, Tomitake Tsukihara, Donglin Bai.

Gap junctions result from the docking of two hemichannels. Depend on the connexin(s) in the hemichannels, homotypic and heterotypic gap junction channels can be formed. Previous chimera studies with domain exchange between different connexins indicated that the extracellular loop2 (E2) is important molecular domain to heterotypic compatibility. Based on the high resolution structure of Cx26 gap junction channel and homology models of heterotypic channels, we analyzed docking selectivity for several hemichannel pairs, and found that the hydrogen bonds between E2 domains are conserved in several heterotypically compatible hemichannels, e.g. between Cx26 and Cx32. According to our model analysis, Cx32 mutation, Cx32N175Y, destroys three hydrogen bonds in the E2-E2 interactions at the heterotypic docking interface. Our model predicts that the Cx32N175Y hemichannel is unlikely to dock with Cx26 hemichannel properly due to steric hindrance at the docking interface. Experimentally, we tagged GFP and RFP to the carboxyl terminals of Cx32 and Cx26 to generate Cx32GFP (Cx32N175YGFP) and Cx26RFP, respectively. Our data showed that tagged Cx26 and Cx32 were able to traffic to cell interfaces forming gap junction plaque-like structures in transfected HeLa/N2A cells. However, Cx32N175YGFP exhibited mostly intracellular distribution and rarely seen in cell-cell interfaces. Double patch clamp analysis demonstrated that this Cx32 mutant did not form functional homotypic channels. When wild-type or mutant Cx32GFP expressing cells were cocultured with Cx26RFP expressing cells, Cx32GFP and Cx26RFP were frequently colocalized at the cell-cell interfaces and formed functional Cx32/Cx26 heterotypic channels. No colocalization was found at the cell-cell interfaces between Cx32N175Y and Cx26 expressing cells, also no functional Cx32N175Y/Cx26 heterotypic channels were identified. Our modeling and experimental data indicate that N175 of Cx32 is a critical residue for heterotypic docking between Cx32 and Cx26 hemichannels.

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Calcium Permeability of Purified and Reconstituted Hemichannels formed by Connexin 26 and the Deafness-Causing Mutant R75W

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Gap-junctional channels are connexin oligomers (dodecamers) formed by head-to-head docking of two hemichannels (each one a connexin hexamer). Mutations of connexin 26 (Cx26) are the most frequent cause of genetic deafness. A single amino-acid mutation at position 75 (R75W) causes autosomal dominant deafness, and we have shown that this mutant is incapable of forming gap-junction channels, but forms functional hemichannels. Here, we studied the permeability of purified Cx26 and R75W hemichannels to Ca^{2+} and large hydrophilic solutes to further our understanding of the deafness associated with R75W expression. Cx26 and R75W were purified based on the affinity for Co^{2+} of a poly-histidine tag fused at the C-terminal end of the proteins overexpressed using the insect cell/baculovirus expression system, and the Cx26 and R75W affinity for the strong cation exchanger Mono S. Essentially all Cx26 and R75W solubilized in dodecylmaltoside were hexamers, and were functional when reconstituted in liposomes, as demonstrated by sucrose- and fluorescent-probe permeability assays. Cx26 and R75W hemichannels were permeable to sucrose, Alexa Fluor 350 and Cascade Blue, but not to Calcein, Fluo-5N or Alexa Fluor 647. Ca^{2+} permeability was evaluated in liposomes containing Cx26 or R75W hemichannels. In the proteoliposomes with the low-affinity calcium-sensitive probe Fluo-5N trapped inside, intra- and extra-liposomal free calcium equilibrated rapidly upon increasing free-[Ca^{2+}] from $<$ 10 nM to ~400 μM. These studies directly show permeability of Cx26 and R75W hemichannels to Ca^{2+}, but failed to identify permeability differences between hemichannels formed by Cx26 and the deafness-causing mutant. This work was supported in part by NIH grants R01GM79629 and R21DC007150, American Heart Association Grant-in-Aid 0755002Y, and a grant from the Center for Membrane Protein Research of TTUHSC.

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Origin and Dynamics of Calcium Waves in the Islet of Langerhans

Richard K.P. Benninger, Troy Hutchens, W. Steven Head, David W. Piston.

Interactions between cells in the islet of Langerhans are critical for the regulation of insulin secretion. Here, we study the electrical coupling and electrical dynamics between β-cells in the islet. We focus on quantitatively measuring multi-cellular calcium oscillations and waves, describing the emergence of these dynamics with a multi-cellular mathematical model, and experimentally testing predictions given by this model.

A key prediction is that calcium waves emerge from sub-regions of the islet with elevated excitability, due to endogenous β-cell heterogeneity in cellular excitability [Benninger et al. *Biophys.J* **95:**p5048 (2008)]. To test this we utilize a 2-channel microfluidic device [Rocheleau et al. *PNAS* **101:**p12899 (2004)] which allows a precise pattern of glucose stimulation to be applied. If glucose is elevated on one side of the islet, calcium waves consistently originate from this region, as predicted. Subsequently, upon uniform glucose stimulation, the wave direction is independent of where glucose was initially elevated: consistent with an intrinsic β-cell heterogeneity that determines calcium wave propagation.

We further explore the balance of β-cell excitability and coupling by describing the calcium activity upon a glucose gradient. In normal islets, a sharp transition in calcium activity occurs midway across the islet. In the absence of gap junction coupling this transition point is shifted towards the quiescent side of the islet. Interestingly following elevated glucose, the initial calcium elevation propagates further across the islet than subsequent calcium waves. We can explain this given a measured glucose dependence of gap-junction coupling.

This gives us further insight into the role of electrical coupling in determining the overall spatiotemporal electrical response. Furthermore the application of mathematical models to predict phenomena which can be experimentally verified to yield new functional insight is a significant advance in understanding emergent multi-cellular behavior.

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Transcriptional Suppression of Connexin 43 by Tbx18 Undermines Cell-Cell Electrical Coupling in Postnatal Cardiomyocytes

Nidhi Kapur, Eduardo Marbán, Hee Cheol Cho.

During embryonic development, critical steps in cardiac lineage specification are guided by T-box transcription factors. Mesenchymal precursor cells expressing Tbx18 give rise to the heart's pacemaker, the sinoatrial node (SAN). We sought to identify targets of Tbx18 transcriptional regulation in the heart by forced adenoviral overexpression in postnatal cardiomyocytes. Monolayers of neonatal rat cardiomyocytes (NRCMs) transduced with GFP alone showed sarcolemmal, punctate Cx43 expression. In contrast, Tbx18-transduced NRCMs exhibited sparse Cx43 expression. Both the transcript and protein levels of Cx43 were greatly downregulated within 2 days of Tbx18-transduction. Injection of Tbx18 in the guinea pig heart *in vivo* markedly suppressed Cx43 expression similar to what was seen in Tbx18-NRCMs. The repressor activity of Tbx18 on Cx43 was highly specific: protein levels of Cx45 and Cx40, which comprise the main gap junctions in the SAN and conduction system, were unchanged by Tbx18. A reporter-based promoter assay demonstrated that Tbx18 directly represses the Cx43 promoter. Phenotypically, Tbx18-NRCMs exhibited slowed calcein dye transfer kinetics (421 ± 54 vs. control 127 ± 43 ms). Intracellular Ca^{2+}-oscillations in control NRCMs monolayers were highly synchronized. In contrast, Tbx18-overexpression led to episodes of spontaneous, asynchronous Ca^{2+}-oscillations demonstrating reduced cell-cell coupling. The decreased electrical coupling led to slow electrical propagation; conduction velocity in Tbx18-NRVMs slowed by more than 50% relative to control (4 ± 1 vs 9 ± 2 cm/s). Taken together, Tbx18 specifically and directly represses the transcript and protein levels of Cx43 in NRCMs *in vitro* and in adult ventricular myocardium *in vivo*. Cx43 suppression led to significant electrical uncoupling, but the preservation of other gap junction proteins (Cx45 and Cx40) permitted action potential propagation at slower velocity. Thus, Tbx18 overexpression recapitulates a key phenotypic hallmark of the SAN, namely the characteristic loose electrical coupling.