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Store-operated Ca^{2+} Entry In Skeletal Muscle Can Be Activated And Deactivated Within Milliseconds Of Ca^{2+} Release And Store Refilling

Bradley S. Launikonis[1], Joshua N. Edwards[1], Fredric von Wegner[2], Oliver Friedrich[1].
[1]University of Queensland, Brisbane, Australia, [2]Johann Wolfgang Goethe University, Frankfurt, Germany.

Store-operated Ca^{2+} entry (SOCE) is a mechanism that allows the entry of Ca^{2+} upon depletion of the internal stores. The skeletal muscle cell is built for the rapid delivery of Ca^{2+} to the contractile proteins. The cell microarchitecture allows this with the surface membrane invaginating into the cell forming the tubular (t-) system which apposes the sarcoplasmic reticulum (SR) for rapid signalling. In skeletal muscle SOCE has been shown to occur within 1 s of Ca^{2+} release (Launikonis & Rios, 2007) but this should be significantly faster if the molecular agonists are prepositioned for activation. To examine SOCE kinetics we used skinned fibres from C57 mice (7-20 weeks old) with t-system trapped fluo-5N, bathed in an internal solution with 50 μM rhod-2. These dyes were simultaneously imaged in xyt mode on a confocal microscope (2 ms/line) while Ca^{2+} release was induced by lowering [Mg^{2+}]. Global Ca^{2+} release induced SOCE activation and deactivation as the Ca^{2+} store refilled upon release inactivation (Launikonis & Rios, 2007). We also observed Ca^{2+} waves in the continued presence of low Mg^{2+}. These waves allowed an accurate observation of the latency between SR Ca^{2+} release and SOCE. Thus SOCE ''coupling delay'' following the initiation of SR Ca^{2+} release was determined to be 27 ± 4 ms (n = 6). SOCE deactivation already started to occur when myoplasmic Ca^{2+} levels dropped only by 10 % suggesting an intact switch-off signal for SOCE from the store. Also, SOCE deactivation rate depended upon SR Ca^{2+} refilling rate in a sigmoidal manner, indicating that binding of luminal Ca^{2+} to Stim1 effectively decoupled from Orai1 instantly during refilling. This suggests conformational coupling between Stim1 and Orai1 mediate SOCE.

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Down And Out. The Functional Effects Of Silencing Calsequestrin 1 Or Deleting Both Calsequestrin Genes In Mammalian Muscle

Leandro Royer[1], Sandrine Pouvreau[2], Ying Wang[3], Gerhard Meissner[3], Jingsong Zhou[1], Pompeo Volpe[4], Alessandra Nori[4], Robert Fitts[5], James W. Bain[6], Feliciano Protasi[7], Paul D. Allen[8], Bjorn Knollmann[9], Danny A. Riley[6], Eduardo Rios[1].
[1]Rush University, Chicago, IL, USA, [2]Universite Claude-Bernard Lyon 1, Lyon, France, [3]University of North Carolina, Chapel Hill, NC, USA, [4]Universita d. S. di Padova, Padua, Italy, [5]Marquette University, Milwaukee, WI, USA, [6]Medical College of Wisconsin, Milwaukee, WI, USA, [7]CeSI-Univ. G. d'Annunzio of Chieti, Chieti, Italy, [8]Brigham and Women's Hospital, Boston, MA, USA, [9]Vanderbilt University, Nashville, TN, USA.

Calsequestrins 1 and 2 are major calcium binding proteins of the SR in skeletal and cardiac muscle. We transiently suppressed synthesis of CSQ1 in fast twitch muscle of live adult mice by transfection with a plasmid coding for siRNA and a marker. Immunoblots showed reduction of CSQ1 by 40 to 100% in the whole treated (FDB) muscle. Ca^{2+} transients and Ca^{2+} release flux were measured in fibers selected for their high expression of the marker and patch clamped. Similar studies were done with FDB fibers from a double-null strain created by crossing CSQ1-null (Paolini et al. 2007) with CSQ2-null mice (Knollmann et al. 2006). Total Ca^{2+} releasable by maximal prolonged depolarization was decreased by up to 30% in silenced and 40% in KO muscles compared with the wild type. The reduction in CSQ had subtle kinetic consequences. The time course of release flux induced by long depolarization lost a ''shoulder'' (present in the WT; Royer et al. 2008). This shoulder reflects a component of the SR Ca^{2+} buffering power characterized by its dependence on $[Ca^{2+}]_{SR}$. Its loss here identifies the shoulder as a kinetic signature of the presence of CSQ. The KO presents additional anomalies, including asynchronous activation of different regions, and, occasionally, abnormally high initial release flux. Both features may be associated with structural changes like those found in the CSQ1 KO (Paolini, 2007). In conclusion, muscle either transiently or permanently devoid of CSQ is still capable of releasing large quantities of Ca^{2+}. Means of Ca^{2+} storage unrelated to CSQ appear to play a major role in skeletal muscle. Supported by NIAMS/NIH.

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Amyloid-β protein impairs Ca^{2+} release and contractility in skeletal muscle from Inclusion Body Myositis mice

Alexander Shtifman[1], Christopher W. Ward[2], Derek R. Laver[3], Jose R. Lopez[4], Noriaki Ikemoto[5].
[1]St. Elizabeth's Medical Center / Tufts University School of Medicine, Boston, MA, USA, [2]University of Maryland, Baltimore, MD, USA, [3]University of Newcastle, Callaghan, Australia, [4]Brigham & Women's Hospital, Boston, MA, USA, [5]Boston Biomedical Research Institute, Watertown, MA, USA.

Inclusion Body Myositis (IBM), the most common muscle disorder in the elderly, is partly characterized by an abnormal, intracellular accumulation of β-amyloid precursor protein (βAPP) and β-amyloid epitopes. The present study examined the effects of β-amyloid accumulation on contractilioty and Ca^{2+} release in skeletal muscle from transgenic mice harboring human βAPP and assessed the consequence of $A\beta_{1\text{-}42}$ modulation of the ryanodine receptor Ca^{2+} release channels (RyR). Muscle from βAPP-transgenic animals produced less peak force, yet fatigued at slower rate than the non-Tg muscle. Analysis of sarcoplasmic reticulum (SR) Ca^{2+} release showed that transgenic myofibers consistently exhibited Ca^{2+} transients with smaller amplitude compared to the non-Tg cells. Although Ca^{2+} removal was slower in transgenic muscle, experiments with SR vesicles in the presence of synthetic β-amyloid peptide ($A\beta_{1\text{-}42}$) did not reveal an acute effect of this peptide on SR Ca^{2+} ATPase. To determine whether modification of RyRs by β-amyloid underlie the effects observed in muscle, in vitro Ca^{2+} release assays and RyR reconstituted in planar lipid bilayer experiments were conducted in the presence of synthetic $A\beta_{1\text{-}42}$. Application of $A\beta_{1\text{-}42}$ to resulted in modification of RyR properties in bilayers, while addition of $A\beta_{1\text{-}42}$ to the SR vesicles resulted in RyR-mediated Ca^{2+} release. These data may relate βAPP mismetabolism in IBM to altered RyR-mediated Ca^{2+} release and muscle contractility.

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Bin1, A Bar Domain Protein, Is Necessary For The Maintenance Of T-tubule Structure And Intracellular Ca^{2+} Homeostasis In Skeletal Muscle

Andoria Tjondrokoesoemo[1], Shinji Komazaki[2], Christopher Ferrante[1], Jingsong Zhou[3], Jianjie Ma[1], Noah Weisleder[1].
[1]UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ, USA, [2]Saitama Medical College, Saitama, Japan, [3]Rush University, Chicago, IL, USA.

Efficient intracellular Ca^{2+} homeostasis in skeletal muscle requires establishment and maintenance of an intact triad junctional complex. Bin1, an amphiphysin family protein contains a conserved BAR domain that facilitates membrane curvature. Bin1 also contains a unique phosphatidylinositol-4,5-bisphosphate (PIP_2) binding domain that is required for transverse-tubule membrane biogenesis during C2C12 myogenic cell differentiation. The lethality associated with Bin1 knockout limits the opportunities to study the function of Bin1 in adult skeletal muscle. Using *in vivo* electroporation to deliver shRNA against Bin1, we knockdown Bin1 expression in the FDB muscle of adult mice. We find that transient loss of Bin1 alters the properties of Ca^{2+} sparks induced by osmotic stress. Specifically, we observe that the mean frequency of Ca^{2+} sparks per minute is reduced in fibers that exhibit a high level of Bin1 knockdown (33.1 ± 5.7), compared to control fibers (138.1 ± 21.7). Kinetic analysis of individual Ca^{2+} sparks shows that spark amplitude ($\Delta F/F_0$) is reduced in the Bin1 knockdown fibers (0.67 ± 0.02) when compared to control (0.85 ± 0.01). Bin1 knockdown also alters the full duration at half maximal (FDHM) of Ca^{2+} sparks. A two-exponential decay function fit of the FDHM histograms indicates that, in the control fiber, the corresponding time constants for Ca^{2+} sparks (t_{01}) are 38.0 ± 2.8 ms and 281.3 ± 40.0 ms for Ca^{2+} bursts (t_{02}). The time constant for Ca^{2+} bursts (t_{02}) are significantly reduced in the high Bin1 knockdown muscle fiber (140.9 ± 41.2 ms). Electron microscopy reveals Bin1 knockdown fibers exhibit vacuolation and swelling of t-tubule structures. Thus, alteration of triad junction structure can potentially affect the resting cytosolic Ca^{2+} levels and internal Ca^{2+} stores in Bin1 knockdown fibers.

Motions of the Cell Surface Molecules

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Effect of Energy Depletion and Antimicrobial Peptides on Single Protein Motility in Living *Escherichia coli*

Tabita Winther[1,2], Lei Xu[1,2], Stanley Brown[1,3], Kirstine Berg-Sørensen[4,5], Lene Oddershede[1,2].
[1]Copenhagen University, Copenhagen, Denmark, [2]Niels Bohr Institute, Copenhagen, Denmark, [3]Molecular Cell Biology Molecular Biology Institute, Copenhagen, Denmark, [4]Technical University of Denmark, Copenhagen, Denmark, [5]Department of Physics, Copenhagen, Denmark.

Using optical tweezers the motility of a single outer membrane protein in *E. coli* bacteria, the λ-receptor, was studied. The λ-receptor is a porin that transports nutrients (maltodextrins) across the bacterial membrane. By poisoning the cells with arsenate and azide the bacterial metabolism was stopped. The motility of the exact same λ-receptor was measured before and after energy depletion. After energy depletion there was a significant decrease in the spread of positions

visited. We have thus established that there is a difference in the movement of membrane proteins in living and dead cells. The active component of the motion of the λ-receptor in living cells has been modeled as an artificial temperature, which estimates the energy necessary for this active motion. The influence of antimicrobial peptides (AMPs) on the outer membrane of bacteria was investigated using the mobility of the λ-receptor as a membrane marker. With the growing resistance to antibiotics AMPs are gaining increased interested. Using the AMPs polymyxin B (PMB), and the non toxic derivate polymyxin B nonapeptide (PMBN), we have investigated the influence of AMPs on the outer bacterial membrane. Cells exposed to PMB showed a decrease in the spread of position visited by the λ-receptor upon poisoning. PMBN is known to increase the permeability of the outer membrane with out killing the cells. Exposure to PMBN did, however, not influence the mobility of the λ-receptor.

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Investigating Axonal Outgrowth and Orientation of Neuroblasts through an Alternating Stiffness Substrate

Szu-Yuan Chou[1], Chao-Min Cheng[1], Maarten P. de Boer[1], Chih-Cheng Chen[2], Philip R. LeDuc[1].
[1]Carnegie Mellon University, Pittsburgh, PA, USA, [2]Academia Sinica, Pittsburgh, Taiwan.

At the interface of cell-substrate interactions, substrate elasticities strongly influence the morphology and function of cellular responses. This is important in diverse areas including neural function, metastasis, and heart disease. Cells are subject to mechanical signals in addition to biochemical signals; therefore, understanding the cellular interactions to substrate stiffness and with extracellular matrix is an important step to define how cultured cells respond when grown on materials that have similar characteristics to physiological conditions. To address the effects of localized elasticity, we developed a new method to control the microenvironment through generating a substrate with localized alternating stiffnesses interacting with cells to affect their structural response. This technique was accomplished through first fabricating polymeric microchannels using conventional soft lithography. We made channels either 30 μm or 100 μm wide, and 50 μm deep with poly(dimethylsiloxane) (PDMS) with a 5:1 ratio of base/curing agent. We then poured PDMS with a 30:1 ratio of base/curing agent into the channels and then removed the extra PDMS to produce a level surface. This produced alternating surfaces with elastic modulus of 800 kPa and 200 kPa adjacent to one another. We coated the surfaces with extracellular matrix and seeded neuroblasts onto the systems. We then differentiated them using retinoic acid (20 μM). We found that the neuroblasts had distinct patterns that emerged as they extended processes to and across these alternating stiffness substrates. Depending on the location of the cell body and the direction of the outgrowth when compared to the alternating stiffness interface, the processes would extend forward in different paths. We believe that this approach will enable greater understanding of axon outgrowth as well as provide insight into a variety of diseases linked to cell-ECM-material interactions.

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FRAP and Photoconversion in Multiple Arbitrary Regions of Interest Using a Programmable Array Microscope (PAM)

Guy M. Hagen[1,2], Wouter Caarls[1], Keith A. Lidke[1,3], Anthony H.B. de Vries[1], Cornelia Fritsch[1,4], B. George Barisas[5], Donna J. Arndt-Jovin[1], Thomas M. Jovin[1].
[1]Max Planck Institute for Biophysical Chemistry, Goettingen, Germany, [2]1st Faculty of Medicine, Charles University in Prague, Prague, Czech Republic, [3]University of New Mexico, Albuquerque, NM, USA, [4]University of Sussex, Brighton, United Kingdom, [5]Colorado State University, Fort Collins, CO, USA.

Photomanipulation (photobleaching, photoactivation, or photoconversion) is an essential tool in fluorescence microscopy. Fluorescence recovery after photobleaching (FRAP) is commonly used for the determination of lateral diffusion constants of membrane proteins, and can be conveniently implemented in confocal laser scanning microscopy (CLSM). Such determinations provide important information on molecular dynamics in live cells. However, the CLSM platform is inherently limited for FRAP because of its inflexible raster (spot) scanning format. We have implemented FRAP and photoconversion protocols using a programmable array microscope (PAM). The bleaching or photoconversion patterns are arbitrary in number and shape, dynamic, and adjustable to and by the sample characteristics. We have used multi-spot PAM-FRAP to measure the lateral diffusion of the erbB3 (HER3) receptor tyrosine kinase labeled by fusion with mCitrine on untreated cells and after treatment with reagents that perturb the cytoskeleton or plasma membrane or activate coexpressed erbB1 (HER1, the EGF receptor EGFR). We also explored the versatility of the PAM for photoconversion in arbitrary regions of interest, in cells expressing erbB3 fused with the fluorescent protein dronpa.

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Glycosphingolipids and Non-Raft Phospholipids Exhibit Very Similar Dynamics in Single-Molecule Observations

Kenichi G.N. Suzuki[1], Takahiro K. Fujiwara[2], Akihiro Kusumi[2].
[1]PRESTO-JST, iCeMS, Kyoto Univ., Kyoto, Japan, [2]ICORP-JST, iCeMS, Kyoto Univ., Kyoto, Japan.

The diffusion of typical raft-associated molecules, glycosphingolipids, GM_1, GM_3, GD_{1a}, was observed using high-speed single-particle tracking (HS-SPT) at a temporal resolution of ~6 μs. Each glycosphingolipid molecule was tagged with a 40-nm-gold particle conjugated by the respective Fab antibody or a Cy3-conjugated Fab antibody. At video rate, both probes exhibited the same diffusion behavior, undergoing apparent simple Brownian diffusion at the same effective diffusion coefficient (about 0.2 $\mu m^2/s$). In HS-SPT observations, each glycosphingolipid molecule underwent actin-dependent hop diffusion over the membrane compartment of an average of 110 nm in diameter at an average frequency of once every 25 ms. Surprisingly, this behavior is the same as that of a typical non-raft phospholipid DOPE, qualitatively and quantitatively. These results are at variance with the previous FCS/FRAP studies. In addition, previous investigations concluded that GM_1, labeled either with cholera toxin (CTX) in its headgroup or with Bodipy-FL on the alkyl chain, diffused much more slowly than non-raft phospholipid probes. Here, we found that CTX bound to GM_1 in the plasma membrane diffused as fast as DOPE during the initial 0.5 s after its binding to the membrane, but slowed by a factor of 4 within 10 min after its binding. These results suggest that the slowed diffusion of GM_1 found in previous observations would probably be due to the influences of CTX-induced GM_1 crosslinking or of perturbation by Bodipy-FL on the alkyl chain. These results indicate that in the plasma membrane of non-stimulated cells, glycosphingolipids movements are not slowed by the possible presence of raft, further suggesting that the rafts in the steady-state cells are much smaller than the compartment size (110 nm) and/or short-lived ($<<$25 ms).

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Role of Membrane Domains in Interferon Receptor Signaling: a Single-molecule Study

Anna Pezzarossa[1], Dirk Schaible[2], Tobias Meckel[1], Jacob Piehler[2], Thomas Schmidt[1].
[1]Leiden University, Leiden, Netherlands, [2]University of Osnabruck, Osnabruck, Germany.

Signaling in living cells is largely mediated through multi-protein complexes, and is triggered by recognition of a chemical ligand by membrane receptors at the extracellular side, leading to activation of cytoplasmatic effectors. Thus, understanding the dynamical behavior of receptors in the cell membrane is fundamental to understand the processes of cell signaling. The system we are studying, type I interferon (IFN) receptor, is a member of the cytokine family, which plays a key role in early innate and adaptive immune responses upon infection by pathogens. Different members of the type-1 IFN family elicit differential responses although binding to the same receptor. The latter comprises two subunits ifnar1 and ifnar2. Upon ligand binding a ternary complex is formed and signaling pathways activated. Using single-molecule wide-field fluorescence microscopy we follow receptor diffusion in the plasma membrane of living HeLa cells. Receptor subunits are labeled through post-translational labeling with synthetic dyes (e.g. Cy5) coupled to coenzyme-A. Each of the receptor subunit is transfected and expressed, both separately and simultaneously, in HeLa cells, allowing measurements on single component as well as on the ternary complex formed upon IFN binding. Using correlation analysis we obtained information on receptor diffusion constants. Switching between fast and slow motility and vice versa was observed, and interpreted as association/dissociation of ternary complex. Mutants ligand with different affinity toward ifnar1 and ifnar2 are also tested to probe the effect on ternary complex dynamics. Finally we show that membrane nanostructure is possibly involved in the dynamic behavior of the complex. Our data lead to a kinetic model for receptor assembly which may help to obtain a better understanding of transmembrane signaling.

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Cortical Cytoskeletal Structures Constrain CD36 Receptor Motion at the Cell Surface to Enhance Aggregation and Signaling

Khuloud Jaqaman[1], Hiro Kuwata[2], William Trimble[2], Sergio Grinstein[2], **Gaudenz Danuser**[1].
[1]The Scripps Research Institute, La Jolla, CA, USA, [2]Sick Kids, Toronto, ON, Canada.

CD36 is a key receptor in human macrophages, binding to multivalent ligands such as oxidized LDL and malaria-infected red blood cells. To study the dynamics and aggregation kinetics of CD36 receptors in the plasma membrane, we used single-molecule imaging combined with single-particle tracking and mathematical modeling of individual receptor behavior. We immuno-labeled