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Chromophore Formation of Fluorescence Proteins and Its Application of Developing Ca^{2+} Sensors

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Ca^{2+} regulates numerous biological processes through spatio-temporal changes of cytosolic Ca^{2+} concentration and subsequent interactions with Ca^{2+} binding proteins in living cells. There is a strong need to develop Ca^{2+} sensors capable of real-time quantitative Ca^{2+} measurements in specific subcellular environments without using natural Ca^{2+} binding proteins participating in signaling transduction.[1, 2] Taking advantage of fluorescence proteins (FPs) as a useful tool, we created a series of Ca^{2+} sensors by engineering a sensitive location of single FP with different color.[3] Both spectroscopic properties including extinction coefficient, quantum yield, and pK_a and metal binding properties of engineered FPs were identified with different spectroscopic methods including absorbance, fluorescence, and circular dichroism. The engineered Ca^{2+} sensors exhibit a ratiometric fluorescence and absorbance changes upon Ca^{2+} binding with affinities corresponding to the Ca^{2+} concentration found in the ER (K_d values range from 0.4 - 2 mM). The developed Ca^{2+} sensors have applied to monitor Ca^{2+} changes occurring in various subcellular compartments including ER and mitochondria of various mammalian cells upon response of different agonists. These sensors were engineered into virus transfection system for its application to monitor cellular Ca^{2+} signaling during muscle contraction and neuron events.

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Orai1 Channel And Ca^{2+}-independent Phospholipase A_2 Are The New Determinants Of Proliferation And Migration Of Vascular Smooth Muscle Cells

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Ca^{2+} entry is known to play an important role in proliferation and migration of vascular smooth muscle cells (SMC), but the molecular mechanisms that mediate these processes are far from being understood. Here we introduce Ca^{2+}-independent phospholipase $A_2\beta$ ($iPLA_2\beta$) and Orai1-encoded plasma membrane channel (that mediate store-operated Ca^{2+} entry (SOCE) in non-excitable as well as excitable cells) as new molecular determinants of proliferation and migration of aortic SMC. Patch-clamp, Ca^{2+} imaging, immunocytochemistry and molecular approaches were used in this study. We demonstrated that molecular knock down of either $iPLA_2\beta$ or Orai1 (but not TRPC1) channel resulted in full inhibition of store-operated current and Ca^{2+} entry in primary aortic SMC. Transfection of SMC with siRNA to Orai1 impaired their proliferation: Orai1 knock down resulted in $69 \pm 4\%$ decrease in the number of BrDU positive cells and $60 \pm 1\%$ reduction in the rate of their proliferation. Similar effects were observed in $iPLA_2\beta$-deficient cells ($65 \pm 3\%$ and $71 \pm 4\%$ reductions, respectively). Interestingly, transfection of SMC with antisense to TRPC1 did not affect SOCE, but significantly reduced the rate of cell proliferation (by $48 \pm 4\%$). Orai1 and TRPC1 produced additive effects on proliferation, and knock down of both channels resulted in $76 \pm 8\%$ reduction in the rate of SMC proliferation, consistent with different mechanisms for Orai1and TRPC1-mediated Ca^{2+} entry. Molecular knock down of $iPLA_2\beta$, or Orai1 channel also impaired SMC ability to migrate in response to 20% FBS, high glucose, or SERCA inhibition. These results suggest that SOCE mediated by $iPLA_2\beta$-dependent activation of Orai1 channel in SMC is required for their proliferation and migration, and may be involved in vascular SMC responses and angiogenesis triggered by different physiological and pathological conditions.

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Complexity Of Relationship Between STIM1, $iPLA_2\beta$ And Orai1 Expression, Puncta Formation And SOCE Activation In Native And Heterologous Systems

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STIM1, Orai1 and $iPLA_2\beta$ have been identified as crucial elements of the store-operated Ca^{2+} entry (SOCE) pathway, but the mechanism of their functional interaction and molecular requirements for SOCE activation remain controversial. Here we used high resolution imaging, patch clamp, molecular and pharmacological approaches to study functional behavior and mutual relationship between STIM1, Orai1 and $iPLA_2\beta$ in native cells and heterologous systems in which fluorescently tagged STIM1 and/or Orai1 were over-expressed. We found that STIM1 accumulated in puncta equally well in the presence or absence of Orai1, and STIM1 accumulation in puncta is not sufficient for Orai1 accumulation in the same areas. The normal I_{CRAC} could be activated in STIM1-deficient cells. We further found that the effects of C-terminus of STIM1 may be profoundly different in native cells and in cells in which Orai1 and/or full length STIM1 were over-expressed. Also, we found that while inhibition of $iPLA_2\beta$ caused dramatic impairment of endogenous SOCE in native cells, its effects on SOCE in heterologous expressing systems may be less prominent, and may require higher concentrations of inhibitors. Our new data provide first indications of the potential differences in SOCE between native cells and heterologous systems, and challenge the idea of a direct conformational coupling between STIM1 and Orai1 as a mechanism of puncta formation and SOCE activation in native cells.

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Membrane Depolarization of Skeletal Muscle Cells Induces IL6 and SOCS3 mRNA Expression Through Calcium Dependent Stat3 Phosphorylation

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Physical activity has been implicated as a major stimulus for Interleuquin 6 (IL6) expression and release from skeletal muscle in humans. However, the pathways involved in these processes are not well established. Previously, we have reported that potassium depolarization induces IL6 mRNA expression in cultured rat myotubes, Ca^{2+} being an important second messenger for this expression, possibly acting through CREB, NFkB and AP-1.
In other cells systems, STAT3 is a key protein in signaling mediated by IL6, and it has been implicated in the IL6 autocrine up-regulation. Some recent reports have shown a possible calcium regulation of STAT3 tyr 705 phosphorylation, and others have implied GSK3β as a regulator of STAT3 activation; in any case, the mechanisms for STAT3 regulation have not been demonstrated. We report calcium-dependent regulation of STAT3, induced by membrane depolarization as a result of electrical stimulation of cultured rat myotubes. Two peaks of STAT3 phosphorylation (5 and 60 minutes) were identified, when an external electrical field (400 1 ms pulses at 45 Hz) was applied. When myotubes were stimulated with 40 mM Caffeine, a similar STAT3 activation was observed, indicating that Ca^{2+} is necessary for STAT3 phosphorylation. On the other side, overexpression of the Ca^{2+} chelating protein parvalbumin, not only completely inhibited the activation of STAT3, but also diminished by 30% the basal levels of STAT3 phosphorylation.
We also show some preliminary results depicting inhibition of STAT3 phosphorylation by lithium, a well known GSK3β inhibitor, suggesting that this pathway could be playing a role in STAT3 activation by membrane depolarization.
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Isoproterenol-Enhanced Diastolic Sarcoplasmic Reticulum Ca Leak in Ventricular Myocytes Requires Activation of Nitric Oxide Synthase

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We have previously shown increased cardiac ryanodine receptor (RyR)-dependent diastolic SR Ca leak to be present in heart failure (HF) and in conditions when beta-adrenergic (β-AR) tone is high. This SR Ca leak could contribute to the cause of the observed decreased contractility in HF by limiting SR Ca load. Simultaneously, it could also lead to arrhythmogenic Ca-dependent inward depolarizing current commonly seen in failing hearts. We recently demonstrated that this leak increases in manner dependent on calcium-calmodulin-dependent protein kinase II (CaMKII) and completely independent of either protein kinase A (PKA) activation or an increase in bulk free Ca concentration ([Ca]i). Here we investigate this PKA- and [Ca]i-independent activation of CaMKII. We have found that while stimulating intact myocytes with the β-AR agonist isoproterenol (ISO, 250 nM) the CaMKII-dependent enhancement of SR Ca leak is abolished by treatment of the myocytes with nitric oxide synthase (NOS) inhibitor Nω-Nitro-L-arginine methyl ester (L-NAME, 100 μM). When SR Ca load was matched in each group (156 μM), myocytes treated with ISO alone had significantly higher leak (14.2 ± 2.0 μM) vs. those treated with ISO and L-NAME (3.8 ± 1.4 μM) or those left completely untreated

(8.0 ± 0.91 μM). When SR Ca leak was matched (9.0 μM) we found the SR Ca load necessary to induce that leak was significantly lower in ISO-treated myocytes (91.6 ± 1.9 μM) vs. those treated with ISO and L-NAME (129.4 ± 16.3 μM) or those left untreated (127.4 ± 2.8 μM). This evidence indicates that NOS activation, and therefore generation of nitric oxide, is necessary for ISO-dependent activation of RyR by CaMKII.

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Discrete Proteolysis Of Neuronal Calcium Sensor 1 By μ-calpain Disrupts Calcium Binding

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Neuronal Calcium Sensor-1 (NCS-1) is a high-affinity, low-capacity calcium-binding protein abundantly expressed in many cell types. NCS-1 interacts with the inositol 1,4,5-trisphosphate receptor (InsP3R) and modulates calcium signaling by enhancing InsP3-dependent InsP3R channel activity and intracellular calcium transients. NCS-1 was also found to be a novel binding partner with the chemotherapeutic drug, paclitaxel (taxol), used to treat a variety of tumor types including ovarian, breast, lung, head, and neck cancers. The immediate response of cells to taxol is a further enhancement of the NCS-1 amplification of $InsP_3R$ dependent calcium signaling. Prolonged treatment with taxol triggers μ-calpain dependent proteolysis of NCS-1. Degradation of NCS-1 may be a critical step in the induction of peripheral neuropathy associated with the taxol treatment. To begin the process of designing a strategy that would protect NCS-1 during taxol administration, we treated NCS-1 with μ-calpain *in vitro* and identified the cleavage site by N-terminal amino acid sequencing and MALDI-mass spectroscopy. Using molecular modeling we found that μ-calpain cleavage of NCS-1 occurs within an N-terminal pseudoEF-hand domain. By sequence analysis this pseudoEF-hand domain should be unable to bind calcium. Nonetheless, our results suggest a role for this pseudoEF hand domain in forming and stabilizing the three functional EF hand domains within NCS-1. Using isothermal titration calorimetry (ITC) we found that the loss of the pseudo EF-hand domain of NCS-1 leads to a markedly decreased affinity for calcium. The inability of the μ-calpain treated NCS-1 to bind calcium may explain the reduced calcium signaling in the presence of taxol and may suggest a plausible strategy for therapeutic intervention of peripheral neuropathy in cancer patients undergoing taxol treatment.

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Effect Of (−)-epigallocatechin Gallate (EGCG), A Green Tea Extract, On Excitation-contraction Coupling Of Murine Cardiomyocytes

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Background: Polyphenolic compounds, green tea, reportedly have protective benefit for cardiovascular disease, but the mechanism(s) are unknown. We have recently found that green tea extracts such as (-)-epigallocatechin gallate(EGCG) bind to skeletal and cardiac ryanodine receptor(RyR) Ca^{2+}-release channels. Here we examined the effect of EGCG(1μM, 100nM, 10nM) on cell shortening and Ca^{2+} kinetics in field-stimulated murine ventricular cardiomyocytes loaded with Fura-2AM and on cardiac RyR channels incorporated in lipid bilayers. **Results:** EGCG at 10 nM already maximally increased myocytes fractional shortening(%FS): ECGC 5.6 ± 0.7, N=19, *vs.* Vehicle, 2.2 ± 0.3, N=25, $p<0.01$), so 10 nM was used for all myocyte experiments. Increased contractility was caused by significantly larger Ca^{2+} transients in presence of ECGC(Fura-2 ratio: 0.64 ± 0.15 *vs.* 0.26 ± 0.03, $p<0.01$). Ca^{2+} transient decay kinetics(a measure of SERCA function) and sarcoplasmic reticulum(SR) Ca^{2+} content measured by rapid caffeine application were not significantly altered by ECGC. As a result, EGCG almost doubled the fraction of SR Ca^{2+} content released during each beat(56 ± 6 % *vs.* 32 ± 4%, $p<0.01$), even though EGCG significantly inhibited L-type Ca^{2+} current($p<0.01$). Decay of Ca^{2+} transients during caffeine application was significantly slower(-EGCG 2.31 ± 0.17 sec *vs.* Vehicle 1.72 ± 0.1 sec, $p<0.01$), suggesting that EGCG significantly inhibits Ca^{2+} extrusion via the NaCa exchanger(NCX). EGCG(500nM) enhanced RyR2 single channel activity >30-fold prolonging mean open time 15-fold without altering unitary conductance. EGCG did not alter SR Ca^{2+} loading capacity in the presence of RyR channel blocker ruthenium red. Taken together, these data suggest that EGCG enhances contractility of intact myocytes via its action on RyR channels. SR Ca^{2+} depletion is prevented by EGCG's concomitant inhibition of the NCX. **Conclusions:** EGCG potently modulates cardiac excitation contraction coupling by acting on RyR and possibly on L-type Ca^{2+}-channel and NaCa exchanger. Supported by **R01HL71670, R01HL88635, and R01AR43140**

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Properties and Functions of Store-Operated Calcium Entry in the Developing Nervous System

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Store-operated calcium channels (SOCs) open in response to depletion of calcium stores in the endoplasmic reticulum. These channels are expressed in a variety of tissues including the immune system, vasculature and hepatocytes. The most widely studied and characterized SOC is the Calcium Release-Activated Calcium (CRAC) channel in the immune system. Recent findings indicate that CRAC channels are activated by local interactions between the ER Ca^{2+} sensor, STIM1 and the CRAC channel subunit, ORAI1. Calcium influx through these channels has been shown to play an important role in transcription of inflammatory mediators such as interleukins and cytokines, mediated by the transcription factor NFAT1.

The current study aims at characterizing these channels in the developing nervous system. Calcium imaging experiments demonstrate the presence of store-operated calcium entry (SOCE) with properties similar to that mediated by the CRAC channel. Calcium influx following store-depletion is blocked by La^{3+}, a potent CRAC channel blocker. In addition, 2-APB (0.01–0.02 mM) causes a transient elevation in intracellular calcium followed by a decrease, consistent with that observed in immune cells. Functional studies done using NFAT1 tagged to GFP show translocation of this transcription factor to the nucleus upon calcium entry following store-depletion. Further, we find an increase in the levels of endogenous NFAT-dependent gene expression using Luciferase reporter assays. Altogether, these results provide evidence for the existence of store-operated calcium entry in the developing nervous system and point towards a regulatory role for this pathway in gene-transcription.

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Investigating the Architecture of the CRAC Channel Pore using SCAM

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Calcium channels are vital for numerous cellular processes in all organisms. Among the many classes of calcium channels, the calcium release-activated Ca^{2+} (CRAC) channel, a member of the Store-operated channel (SOC) family is essential for the proper development and maintenance of the immune system, mediating critical functions such as T cells proliferation, release of inflammatory mediators, and motility. This interesting channel has a biophysical fingerprint consisting of an extremely high selectivity for Ca^{2+}, a narrow pore size (3.9Å), a very small unitary conductance, and several modes of modulation. A single amino acid mutation (R91W) results in a loss of CRAC channel function also known as severe combined immunodeficiency (SCID) in human patients. Although the CRAC channel current has been well-characterized, no structural information is known about the channel or its pore.

The objective of this study is to investigate the architecture of the CRAC channel pore using the substituted cysteine accessibility method (SCAM), which has been applied to several ion channel proteins and has provided significant structural insight from topology to conformational changes. Our initial studies analyzed the effects of MTS reagents, cysteine-modifying compounds, on the TM1 and the TM1-TM2 extracellular loop region around E106, an important residue that controls Ca^{2+} selectivity and ion permeation. We find that the ability of MTS reagents to block current diminishes as the residues in TM1-TM2 become more removed from E106. These results suggest that this region of the protein may form the entrance to the channel pore, thus providing the first insight into the architecture of the CRAC channel.

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Both Membrane Depolarization And IL-6 Induce Calcium-Dependent Hsp70 Expression In Skeletal Muscle Cells

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Adaptive response of skeletal muscle to challenges imposed by contractile activity is associated to changes in specific genes expression. IL-6 and Hsp70 are proteins involved in the maintenance of skeletal muscle homeostasis during stress episodes and are markedly expressed in skeletal muscle after physiological contraction. Muscle-derived IL-6 has systemic and local effects acting in a hormone-like fashion, nevertheless the molecular bases of its functional role on skeletal muscle is poorly understood. We have demonstrated that depolarization evoked IP_3 mediated slow calcium transients, associated to cell nuclei are involved in the up-regulation of IL-6 transcriptional activity in skeletal muscle cells.