Calcium Fluxes, Sparks & Waves II

1528-Pos

Polyhistidine Peptide Inhibitor of the Aβ Calcium Channel Potently Blocks the Aβ-Induced Calcium Response in Cells

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Based on the consistent demonstrations that the Aβ peptide of Alzheimer's disease forms calcium permeant channels in artificial membranes, we have proposed that the intracellular calcium increase observed in cells exposed to Aβ is initiated by calcium fluxes through Aβ channels. We have found that a small four histidine peptide, NAHis04, potently inhibits the Aβ-induced calcium channel currents in artificial lipids membrane. Here we report that NaHis04 also potently blocks the intracellular calcium increase which is observed in cells exposed to Aβ. PC12 cells loaded with Fura 2AM show a rapid increase in fluorescence with rapidly return to base line after Aβ is added to the medium. This fluorescence change occurs even when the medium contains nitrendipine, a voltage-gated calcium channel blocker, but fails to occur when application of Aβ is preceded by addition of NAHis04. Steep dose response curves of percentage of responding cells and cell viability show that NAHis04 inhibits in the μm range in an apparently cooperative manner. We have developed numerous models of Aβ pores in which the first part of the Aβ sequence forms a large beta barrel ending at His13. We have modeled how up to four NAHis04 peptides may block these types of pores by binding to side chains of Aβ residues Glu 11, His 13, and His 14.

1529-Pos

Elucidation of Ca^{2+} Influx through Alzheimer's Aβ Channels in the Aβ-Induced Cellular Ca^{2+} Response

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The Alzheimer's disease Aβ peptide interaction with the plasma membrane of cells results in a response characterized by the elevation of the intracellular (cytoplasmic) Ca^{2+} concentration which may critically perturb Ca^{2+} homeostasis. Calcium influx into the cytosol can occur across the plasma membrane via receptor-mediated, voltage-gated, store-operated calcium channels, or from internal stores. Based on the ion channel formation by Aβ peptides on artificial membranes *w*e have proposed that perturbation in Ca2+ homeostasis induced by Aβ could be caused by external calcium entering through ion channels formed by Aβ in the plasma membrane. The activity of these channels would permit the entrance of extracellular calcium ions into the cell, subsequently triggering the release of calcium from internal stores. To elucidate the contribution of calcium influx through the Aβ ion channels to the Aβ-induced calcium response we used specific blockers of plasma membrane channels and specific inhibitors of the mechanisms that permit the release of calcium from the ER, the largest intracellular store. With all those mechanisms blocked and inhibited, we visualized a fast raising, short-lasting calcium entry immediately after PC12 cells were exposed to Aβ. This calcium signal was identified as calcium flowing through the Aβ channels by using specific Aβ channel blockers. The Aβ channel blockers did not affect other mechanisms that contribute to the cytosolic calcium increase, and their use prevented the calcium entry through the Aβ channels and consequent development of the whole cellular Aβ-induced calcium response.

1530-Pos

Functional Analysis of GPCR and Calcium Channel Targets Using Quest Fluo-8

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Calcium flux assays are preferred methods in drug discovery for screening G protein coupled receptors (GPCRs) and calcium channels. Quest Fluo-8 AM, Fluo-3 AM and Fluo-4 AM are evaluated for several GPCR and ion channel targets. They share the same assay principle. All the three fluorogenic calcium indicators are in the form of non-fluorescent AM esters. Once inside cells, the lipophilic AM blocking groups are cleaved by non-specific cellular esterases, resulting in negatively charged fluorescent dyes that stay inside cells, and their fluorescence intensities are greatly enhanced upon binding to calcium. When cells stimulated with bioactive compounds, the receptor signals release of intracellular calcium, which greatly increase their fluorescence signals. In conclusion, Fluo-3 AM, Fluo-4 AM and Quest Fluo-8 AM are robust tools for evaluating GPCR and calcium channel targets and screening their agonists and antagonists with fluorescence microplate readers, fluorescence microscopes or flow cytometers.

1531-Pos

Persistent Calcium Sparklet Activity of L-Type Calcium Channels: Link Between PKC and c-Src

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Ca^{2+} sparklets are a fluorescence signal associated with Ca^{2+} entry through L-type calcium ($Ca_v1.2$) channels, which are primary Ca^{2+} entry pathway in many excitable cells. Ca^{2+} sparklets are quantal in nature (q = 34 nM $\Delta[Ca^{2+}]_i$) and their activity is categorized as low ($0<nP_s<0.2$) or persistent ($nP_s>0.2$), where n = no. of quantal levels and P_s = active sparklet probability. Both types of Ca^{2+} sparklets are present in vascular smooth muscle cells (VSMCs) but only low activity Ca^{2+} sparklets are present in heterologously expressed $Ca_v1.2$ channels unless PKCα is activated. The latter effect presumably requires $Ca_v1.2$ phosphorylation, perhaps at the canonical PKA phosphorylation site, S^{1901}, yet the exact phosphorylation site remains unclear. Using TIRF microscopy in conjunction with whole cell voltage clamp, we detected persistent Ca^{2+} sparklet activity in HEK 293 cells co-expressing $S^{1901}A$ $Ca_v1.2c$ and PKCα, indicating that PKCα does not phosphorylate S^{1901}. Also, persistent Ca^{2+} sparklets were detectable in cells expressing WT $Ca_v1.2c$ in the absence of PKCα if c-Src was co-expressed. Furthermore, Ca^{2+} sparklet activity was reduced in cells expressing WT $Ca_v1.2c$ and kinase dead c-Src (7/9 cells). To test if phosphorylation of a previously identified $Ca_v1.2c$ tyrosine phosphorylation site (Y^{2122}) by c-Src mediated persistent Ca^{2+} sparklet activity, we co-expressed $Y^{2122}F$ $Ca_v1.2c$ and c-Src in HEK cells. Persistent Ca^{2+} sparklet activity was present under these conditions; however, Ca^{2+} sparklet activity was reduced in cells co-expressing $Y^{2139}FCa_v1.2c$ and c-Src. These data suggest that c-Src may phosphorylate $Ca_v1.2c$ at Y^{2139} under basal conditions to produce persistent Ca^{2+} sparklet activity. Future experiments on HEK cells expressing $Y^{2139}FCa_v1.2c$ and PKCα will allow us to determine if persistent Ca^{2+} sparklet activity in VSMCs is evoked by a common mechanism involving PKCα and c-Src.

1532-Pos

Optical Stimulation of Ca^{2+} Transients in Smooth Muscle Cells

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The modulation of intracellular Ca^{2+} plays a huge role in controlling important cell functions such as cell division, signaling, contraction and cell death. To date, intracellular Ca^{2+} dynamics have mainly been investigated using electrophysiological measurement techniques such as patch clamp experiments often used in combination with fluorescence imaging techniques. To activate intracellular channels, drugs are often used. However, because of diffusion and mixing constraints there is little precision in the time course of channel activation and deactivation. In addition, the invasive nature of conventional whole cell patch clamp techniques disturbs the intracellular environment and may so alter channel behaviour.

We report a non-invasive technique that induces controlled Ca^{2+} responses in isolated smooth muscle cells using a holosteric incoherent light source as a stimulus. Using a conventional epi-fluorescence microscope configuration, cells labeled with a Ca^{2+} fluorescent indicator (Fluo-3AM) were stimulated using the low intensity light ($<1.5mW \cong \lambda=488nm$) and the resultant Ca^{2+} transients were visualized using a highly sensitive CCD camera. We will describe the cell stimulation protocol used and present data demonstrating the efficacy of this low cost and minimally invasive technique. We will also describe the investigation into the origin of the light induced Ca^{2+} responses.

1533-Pos

Agonist-Evoked Calcium Wave Progression Requires Calcium and Ip_3 in Smooth Muscle

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Smooth muscle responds to IP_3-generating agonists by producing Ca^{2+} waves to spread information within and between cells. The mechanism of wave progression has been investigated in voltage-clamped single smooth muscle cells. Agonist-evoked waves initiated as a uniform rise in $[Ca^{2+}]_c$ over a substantial length (~30 μm) of the cell. During regenerative propagation, the wave-front was approximately 1/3 the length (~9 μm) of the $[Ca^{2+}]_c$ change at the initiation site. The wave-front progressed at a constant velocity though amplitude varied through the cell. Differences in sensitivity to IP_3 may explain the variation in amplitude; local release of IP_3 evoked $[Ca^{2+}]_c$ increases of varying amplitude in different regions of the cell. The wave-front does not progress by Ca^{2+}-dependent positive feedback alone. In support, colliding $[Ca^{2+}]_c$

increases evoked in response to IP_3, released locally in two parts of the cell, did not annihilate but approximately doubledin amplitude. This result suggests that the $[Ca^{2+}]_c$ increase, generated by local release of IP_3, had not regeneratively propagated but diffused passively from the release site. Notwithstanding, Ca^{2+} was required for IP_3-mediated wave progression to occur. Increasing the Ca^{2+} buffer capacity in a small (2 μm) restricted region of the cell immediately in front of a carbachol-evoked Ca^{2+} wave, by photolyzing the caged Ca^{2+} buffer diazo-2, halted progression at the site of photolysis. Failure of local increases in IP_3 to evoke waves appears to arise from the restricted nature of the IP_3 increase to small areas within the cell. When IP_3 was elevated throughout a localized increase in Ca^{2+} propagated as a wave. Together, these results suggest that waves initiate over a relatively large length of the cell and both IP_3 and Ca^{2+} are required for active propagation of the wave-front to occur.

1534-Pos

Increased Calcium Response to Depolarization in Voltage Clamped Skeletal Muscle Cells of a Transgenic Model of Amyotrophic Lateral Sclerosis

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Mitochondrial Ca uptake is believed to help regulate mitochondrial metabolism and synthesis of ATP to meet the demands of muscle contraction. Whether mitochondrial Ca uptake modifies Ca signaling during EC-coupling remains an open question. While studies show that mitochondria in skeletal muscle may take up Ca during contraction, it is not known whether altered mitochondrial Ca uptake can play a role in pathophysiological conditions. Our study on ALS mouse model G93A shows that ALS muscle fibers display defective mitochondria with loss of their inner membrane potential in fiber segments. The finding of localized mitochondrial defects in ALS fibers presents a unique opportunity to test whether changes in mitochondrial function can affect intracellular Ca signaling, as Ca release activity can be compared in regions with or without depolarized mitochondria in the same muscle fiber. By loading muscle fibers with TMRE (a probe of mitochondrial membrane potential) and fluo-4 (a Ca indicator) we characterized simultaneously mitochondrial function and Ca release activity in living muscle fibers. The fiber segment with depolarized mitochondria shows greater osmotic stress-induced Ca release activity. Abolishing mitochondrial inner membrane potential by FCCP or blocking mitochondrial uniporter by Ru360 exacerbates the osmotic stress-induced hyperactive Ca release. Furthermore, we evaluated the voltage-induced Ca transient by patch-clamping ALS fibers and found that fiber segments with depolarized mitochondria displayed 5~25% greater Ca transients. Our study constitutes a direct demonstration of the importance of mitochondria in shaping cytosolic Ca signaling in skeletal muscle. Malfunction of mitochondrial Ca uptake may play an important role in muscle degeneration of ALS. Supported by MDA/NIH.

1535-Pos

CICR and Calcium-Dependent Inactivation, Quantified Through the Response to Artificial Ca Sparks in Single Muscle Cells

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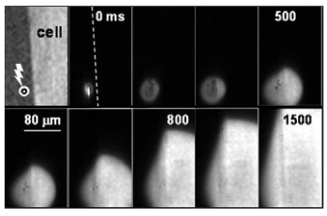
Local calcium stimuli (artificial sparks) generated by 2-photon breakdown of the cage NDBF-EGTA were applied to evoke Ca release from the SR in single skeletal or cardiac muscle cells undergoing fast Ca imaging with the low affinity dye fluo 4FF. The figure shows selected sequential images of the Ca transient generated by a frog skeletal muscle fiber with permeabilized plasmalemma, in response to a spark (elicited *outside* the fiber to avoid photodamage). Two types of responses were observed: (i) an all-or-none wave -shown- that propagates over the entire cell and (ii) graded responses, which fail to propagate. Release analysis (Ríos, JGP 1999; Figueroa, this meeting) separates SR release from simple diffusion of photo-released Ca into cells. The technique yields a sensitive measure of threshold $[Ca^{2+}]$ for release activation, which in the example (0.3 mM $[Mg^{2+}]_{cyto}$) was 1 μM, and can monitor inactivation by combining multiple stimuli. Modeling of these responses aims at describing quantitatively the properties of activation, as well as the roles of inactivation and depletion in the control of Ca release. Other details and acknowledgments are presented elsewhere (Figueroa, this meeting.)



1536-Pos

Flux in Artificial Ca Sparks Generated by 2-Photon Release from a Novel Cage Confocally Imaged at Microsecond Resolution

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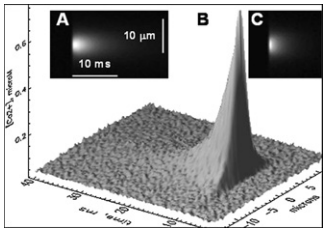
Control of calcium signaling in striated muscle relies on concurrent actions of calcium ions to promote and inhibit release channel opening. To understand these actions we developed artificial Ca sparks generated by 2-photon (2P) release from NDBF-EGTA (Momotake, Nature Methods 2006) as quantifiable local stimuli. A ''Dual Scanner'' (Zeiss) delivers IR laser flashes through a LSM 510 scanner, while rapidly imaging fluorescence of a $[Ca^{2+}]$ monitor via a slit scanner (5-LIVE; *ca* 100 μs/line). Ca sparks of 0.1 to 10 μM (A, B) are elicited in a droplet after microseconds of 2P irradiation at 720 nm and imaged with the low affinity dye fluo 4FF. Reaction-diffusion analysis (Ríos, JGP 1999) yields the flux of Ca photorelease (C). This flux, which initially reaches several hundred mM/s, decays with τ of 2-3 ms. The technique is used to measure physico-chemical properties of calcium ligands, including bio-sensors. Applied inside muscle fibers (Figueroa, this meeting) it serves to quantitatively characterize calcium control in cells.



Instrument purchased with a S10 NCRR award and Hasterlik Family matching funds. Supported by NIAMS, NHLBI/NIH and MDA.

1537-Pos

Effects of High [BAPTA] Inside Mouse Muscle Fibers Reveal a Role of Calcium in the Termination of Voltage-Operated Calcium Release from the SR

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Striated muscles have a termination mechanism that causes the flux of Ca release from the SR, whether activated by an action potential or a voltage pulse, to rapidly cease after an early peak. This mechanism is viewed as a fundamental property that insures stability and ''gradedness'' in the control of Ca signals. To probe this termination, the evolution of Ca release flux was derived from cytosolic Ca transients elicited by SR-depleting depolarizations of long duration, in voltage-clamped cells of mouse FDB muscle. In the presence of 5 mM of the Ca buffer BAPTA, the release flux underwent major changes compared with a 10 mM [EGTA] ''reference'' situation (studied by Royer, J Physiol 2008). Ca release reached an early peak and then decayed to a sustained phase that was higher and briefer than in reference, often including a second rise or ''hump''. In BAPTA, measurable release only lasted 100 ms or less. Its time integral -which measures the SR Ca content releasable by depolarization_was on average 1.4 mM (n=12 cells), compared with 2.1 mM for 18 cells in reference.

An increase in flux with conserved releasable content indicates that BAPTA promotes flux and hastens emptying of the SR without greatly changing its storage properties (including lumenal $[Ca^{2+}]$). A greater Ca flux driven by a similar $[Ca^{2+}]$ gradient requires greater and/or more sustained channel openness. These observations suggest the presence of a release channel-inhibiting mechanism (CDI) mediated by binding of cytosolic Ca^{2+} to open or closed channels, a mechanism more susceptible to interference by BAPTA than the slower-reacting EGTA. Work funded by NIAMS/NIH and an MDA grant to Dr. Jingsong Zhou, who we thank for continued support.

1538-Pos

D4cpv-Casq1. A Novel Approach for Targeting Biosensors Yields Detailed Dynamic Imaging of Calcium Concentration Inside the Sarcoplasmic Reticulum of Living Cells

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Dynamic imaging of $[Ca^{2+}]$ inside the SR of skeletal muscle is hampered by the limited sensitivity of available ratiometric biosensors (Rudolf, JCB 2006) and faces difficulties of calibration when using non-ratiometric dyes (Kabbara and Allen, J Physiol 2001). Impressed by the apparently perfect targeting of