IP3 Receptors

2655-Pos

Novel Insights into the Role of Junctate in Calcium Homeostasis: Identification of Binding Domain on the InsP3R and Cellular Localization as Determined by TIRF Microscopy

Susan Treves[1,2], Johanna Griesser[1], Clara Franzini-Armstrong[3], Michael X. Zhu[4], Francesco Zorzato[1,2].
[1]Basel Unoversity Hospital, Basel, Switzerland, [2]University of Ferrara, Ferrara, Italy, [3]University of Pennsylvania, Philadelphia, PA, USA, [4]Ohio State University, Columbus, OH, USA.

Junctate is a 33 kDa calcium binding protein with a single ER/SR membrane spanning domain expressed in excitable and non-excitable tissues. It is generated by alternative splicing from the AßH-J-J locus, which also encodes the enzyme aspartyl-ß-hydroxylase, the sarcoplasimc reticulum structural protein junctin and humbug, a truncated version of aspartyl-ß-hydroxylase, lacking its catalytic domain, which shares with junctate the high capacity moderate affinity Ca2+ binding domain and is over-expressed in a variety of tumours. We have previously shown that junctate forms a macromolecular complex with the InsP3R and TRPC3 channels and when transiently over-expressed in HEK293 cells, it induces extensive proliferation of the ER resulting in significantly larger and more frequent couplings between the ER and the plasma membrane. In the present work we have mapped the binding domain of the cytoplasmic NH2 terminus of junctate on the InsP3R and show that it binds to the domain involved in InsP3 binding. Such a result is supported by the finding that in the presence of a peptide encompassing the NH2 terminal domain of junctate, the Bmax for InsP3 binding is significantly higher than that obtained in the presence of an unrelated peptide. Transmission electron microscopy revealed that clones stably transfected with junctate-YFP display a significant larger number of junctions between the ER and the plasma membrane compared to control HEK293 cells and this effect is enhanced in clones that also over-express TRCP3. The size and distribution of these punctae however, was not affected by the addition of agonists mobilizing calcium via InsP3R activation.

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Visualizing Alpha Helices in the Transmembrane Region of IP_3R1 Calcium Release Channel by Single Particle Electron Cryomicroscopy

Steven J. Ludtke[1], Thao P. Tran[2], Que T. Ngo[2], Vera Y. Moiseenkova-Bell[3], Joshua T. Maxwell[4], Gregory A. Mignery[4], Wah Chiu[1], **Irina I. Serysheva**[2].
[1]Baylor College of Medicine, Houston, TX, USA, [2]The University of Texas Health Science Center at Houston Medical School, Houston, TX, USA, [3]Case Western Reserve University School of Medicine, Cleveland, OH, USA, [4]Stritch School of Medicine, Loyola University Medical Center, Maywood, IL, USA.

Type 1 inositol 1,4,5-trisphosphate receptor (IP_3R1), a 1.3 MDa tetrameric membrane protein regulates the release of Ca^{2+} from endoplasmic reticulum stores into the cytoplasm and plays an essential role in a wide variety of cellular processes. We determined the structure of IP_3R1 in the closed conformation at ~9-Å resolution by using single particle electron cryomicroscopy. The channel protein was solubilized with detergent from rat cerebellar microsomes and purified by immunoaffinity chromatography. The purified channels were reconstituted into Ca^{2+}-loaded lipid vesicles and released Ca^{2+} in response to nanomolar concentrations of IP_3, indicating their functionality. Isolated IP_3R1 particles were embedded in vitreous ice for cryo-EM in the presence of 1 mM EGTA to drive the channel protein into its closed conformation. The 3D density map of IP_3R1 was generated with 37,231 particles extracted from 929 CCD frames collected on a JEOL2010F electron cryomicroscope. The reconstruction was performed using EMAN software. This structure allows visualizing a number of alpha-helices in the membrane-spanning region of the IP_3R1 channel, including the inner alpha-helices lining the tapering ion conduction pathway. The molecular architecture of the closed pore is established based on the 9-Å cryo-EM density map of IP_3R1 and via computational and bioinformatics approaches.
This research is supported by grants from NIH (R01GM072804, P41RR02250) and MDA Research Grant 88677.

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Spatial Modeling of IP3 Channel Clusters and Calcium Puffs

Sten Ruediger[1], Jian-Wei Shuai[2].
[1]Humboldt University, Berlin, Germany, [2]Xiamen University, Xiamen, China.

Using detailed numerical analysis as well as strongly reduced models we study release of calcium from clusters of IP3 receptor channels. In the first part we present hybrid stochastic-deterministic simulations of release from a cluster of nine channels. We adopt recent advances in imaging of calcium release, which showed a considerable spatial separation of channels. We find that, due to the separation of channels and the three-dimensional transport of calcium away from the source area, the calcium concentration is generally heterogeneous in the cluster area. Based on a Markovian description of channel gating and a fitting of ligand/channel reactions to single-channel data, we obtain puffs that strongly resemble recent recordings in neuroblastoma cells. We conclude that spatial heterogeneity is crucial part of the understanding of puffs. In a second part of this work we take up the issue of deriving a reduced model in terms of a discrete or continuous description of gating variables. We argue that lack of homogeneity in [Ca2+] obtained in the detailed simulations obliterates the assumption of mixing of reactants (here ligands and channels) and thus the validity of the law of mass action. Effective reaction kinetics can be derived, however, by distinguishing concentrations of self-feedback of channels and coupling to different channels, thus eliminating detailed balance. We infer a minimal Markovian model as well as a corresponding Langevin model. Importantly, only the Markovian description reproduces calcium puffs, while a Langevin model wrongly predicts a stationary regime of high inhibition. The analysis of the Markovian model allows further insight into the functioning of calcium puffs.

2658-Pos

Role of Agonist-Independent Conformational Transition (AICT) in I P3 R Cluster Behavior

Divya Swaminathan, Peter Jung.
Ohio University, Athens, OH, USA.

Local intracellular calcium (C a^{2+}) signals arise due to the release of C a^{2+} ions from internal stores into the cytosol through small clusters of inositol-1,4,5-triphosphate (I P_3) receptors. To explain single I P_3 receptor open probability data from nuclear patch clamp experiments, theoretical simulations have favored the existence of an agonist-independent conformational transition(AICT) from closed to an open state. We present results from a computational study wherein we explore the impact of the proposed agonist independent conformational transition on the collective release of calcium from I P_3 R clusters. A wealth of experimental data profiles collective cluster release. Our results show that consistency of cluster release between theory and experiments in fact constrains the kinetics of the agonist-independent conformational transition (AITC) to values which lead to small open probabilities for a single I P_3 receptor, inconsistent with nuclear patch clamp experimental data.

2659-Pos

Global Dynamic Conformational Changes in the Suppressor Domain of IP_3 Receptor by Stepwise Binding of the Two Lobes of Calmodulin

Yonghyun Song, Sunmi Kang, Hyeji Yang, Chang Kook Suh, Sunghyuk Park.
School of Medicine, Inha University, Incheon, Republic of Korea.

The roles of calmodulin (CaM) has been a key point of controversy in the regulation of inositol-1,4,5-trisphosphate receptor (IP_3R). Current views differ in terms of the involvement of CaM as calcium sensors and calcium's functions in the process. To help resolve these issues, we studied the interaction between CaM and the suppressor domain of IP_3R, a key allosteric regulatory domain, in the absence and presence of calcium. Through NMR binding experiments, we observed dramatic peak disappearances of the suppressor domain upon interaction with apo-CaM. These data indicated that apo-CaM induces large-scale dynamic conformational changes in the suppressor domain, most probably involving partial unfolding and sub-domain rearrangement. Resonance assignments of CaM surprisingly revealed that its C-lobe alone can cause these changes. Subsequent NMR binding experiments showed that calcium allows the free N-lobe to additionally bind to the suppressor domain, which induces extra conformational changes in both of the proteins. Our data also suggest that the extra changes in the suppressor domain are secondary to those in calmodulin. Based on these results, we propose that apo-CaM, through its C-lobe, can prime the allosteric regulation by partially unfolding the suppressor domain, which could be propagated to distant sites to open inhibitory calcium binding sites. Calcium then could bind to the CaM's N-lobe and the inhibitory binding sites in IP_3R, eliciting additional conformational changes and actual inhibition of IP_3R. We believe that our results reconcile previous allosteric models and provide new insights in the mechanism of calcium/CaM-mediated regulation of IP_3R.

2660-Pos

An Analysis of the Activity of Two Disease Associated Mutations of the Inositol 1,4,5-Trisphosphate Receptor Type-1

Tricia Nienaber, Matthew J. Betzenhauser, Larry E. Wagner, David I. Yule.
University of Rochester, Rochester, NY, USA.

Inositol 1,4,5-trisphosphate receptor type 1 ($InsP_3R1$) is abundantly expressed in the central nervous system. $InsP_3R1$ functions to release Ca^{2+} from the endoplasmic reticulum (ER) upon stimulation by inositol 1,4,5- trisphosphate

($InsP_3$). Recently, perturbations in the $InsP_3R1$ receptor have been linked to a human neurodegenerative disorders. The slow, progressive neurological disease, Spinocerebellar Ataxia type15 (SCA15), is inherited through an autosomal dominant gene and causes degeneration of the cerebellum. A missense mutation P1059L in the regulatory and coupling domain of the receptor (P1073L in mice) has been suggested by linkage analysis to result in SCA15 in humans. A further mutation, Itpr1Δ18/Δ18, causes the deletion of 6 amino acids in $InsP_3R$-1 and results in an ataxic phenotype in mice. We have created stable cell lines expressing corresponding mutations in the rodent $InsP_3R1$ gene in DT40-3KO cells, an unambiguously $InsP_3R$ null background. Immunoblotting confirmed expression of the mutant channels at comparable levels to wild-type. In both "on-nuclear" single channel patch clamp experiments and Ca^{2+} imaging both mutated $InsP_3R1$ receptors are functional Ca^{2+} channels. A comparison of the activity of the mutated receptors with wild type $InsP_3R1$ will be presented.

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Regulation of Inositol 1,4,5 Trisphosphate Receptors by InsP3 Receptor-Associated cGMP Kinase Substrate (IRAG)

Wataru Masuda, Matthew J. Betzenhauser, David I. Yule.
University of Rochester, Rochester, NY, USA.

Various factors interact with IP_3R, regulating Ca^{2+} release and thus serve to define the spatial and temporal characteristics of the cytosolic Ca^{2+} signal. IP_3R-associated cGMP kinase substrate (IRAG) has been reported to bind IP_3R type-1 and inhibit Ca^{2+} mobilization in smooth muscle cells. No information is however available as to whether IRAG interacts or has functional effects on other IP_3R family members. In this study, we examined whether IRAG binds to and regulates Ca^{2+} release via IP_3R type-2 or type-3. cDNA encoding IP_3R type-1, IRAG-GFP, and protein kinase G1β (PKG1β) were transiently transfected into COS cells. Following immunoprecipitation from cell lysates with an anti-GFP antibody, IP_3R type-1 was detected by immunoblotting. In contrast, an IRAG-GFP construct (IRAGΔE12-GFP) in which 40 amino acids required for binding with IP_3R was deleted, failed to interact with IP_3R type-1, but was still capable of binding to PKG1β , an additional cognate binding partner of IRAG. Similarly, IP_3R type-2 or IP_3R type-3 could be shown to interact with IRAG-GFP but not IRAGΔE12-GFP in COS cells. Next, we investigated if IRAG regulates IP_3-induced Ca-release using DT40-3KO cell lines stably expressing mammalian IP_3R type-2 or type-3 in isolation. In DT40-3KO cells stably expressing IP_3R type-2, and transiently expressing Muscarinic M3 receptor, IRAG-GFP and PKG1β, cell permeable PKG activators reduced the muscarinic agonist carbachol (CCh)-induced Ca^{2+}-release. Ca^{2+} oscillations induced by low concentrations of CCh or by stimulating the endogenous B cell receptor were similarly attenuated. No inhibitory effect was evident in cells expressing IRAGΔE12-GFP or in the absence of IRAG-GFP. Similar results were obtained with DT40-3KO cells stably expressing IP_3R type-3. These results indicate that Ca^{2+} release through all Inositol 1,4,5 trisphosphate receptors are inhibited by an interaction with IRAG and PKG1β.

2662-Pos

CaMKII-Mediated Phosphorylation of $InsP_3R2$ at Serine-150 Results in Decreased Channel Activity

Joshua T. Maxwell, A.S. Aromolaran, Gregory A. Mignery.
Loyola University Medical Center, Maywood, IL, USA.

$InsP_3$ mediated calcium release through the type-2 inositol 1,4,5-trisphosphate receptor ($InsP_3R2$) in cardiac myocytes results in the activation of associated CaMKIIδ (Bare et al, 2005, *JBC*; Wu et al, 2006, *JCI*), enabling the kinase to act on downstream targets, such as histone deacetylases 4 & 5 (HDAC4 & HDAC5) (Zhang et al, 2007, *JBC*). The CaMKII activity also feedback modulates $InsP_3R2$ function by direct phosphorylation and results in a dramatic decrease in the receptor-channel open probability (P_o). The results of this study show that in planar lipid bilayers the channel activity of $InsP_3Rs$ can be inhibited by CaMKII-mediated phosphorylation, and that effect can be reversed by addition of protein phosphatases. Furthermore, we have used fragments of the $InsP_3R2$ and site-directed mutagenesis to determine that Serine at residue 150 is the CaMKII phosphorylation site responsible for modulation of channel activity. Non-phosphorylatable (S150A) and phospho-mimetic (S150E) mutations were constructed in the full-length $InsP_3R2$, expressed in COS cells and studied in planar lipid bilayers. Upon treatment with CaMKII, the non-phosphorylatable channel showed no decrease in activity. Conversely, the phosphomimetic channel displayed a very low P_o under normal recording conditions in the absence of CaMKII (2μM $InsP_3$ and 250nM $[Ca^{2+}]_{FREE}$), thus mimicking a channel that has been phosphorylated by CaMKII. The results of this study show that Serine-150 of the $InsP_3R2$ is phosphorylated by CaMKII and results in a decrease in the channel's open probability. The mechanism for the regulation of the $InsP_3R2$ appears to be a consequence of altered affinity for $InsP_3$ at the ligand binding site and/or perturbation of the receptor amino to carboxyl-terminal interaction and is currently being examined.

These studies were supported by National Institutes of Health grant PO1HL080101.

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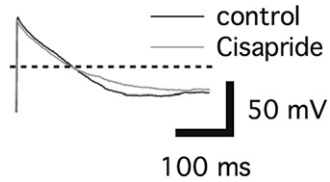
Excitement Over Automated Patch Clamp: Action Potentials from Cardiac Myocytes

Sonja Stoelzle[1], **Andrea Bruggemann[1]**, David Guinot[1],
Alison Haythornthwaite[1], Michael George[1], Cecilia Farre[1],
Claudia Haarmann[1], Ralf Kettenhofen[2], Niels Fertig[1].
[1]Nanion Technologies, Munich, Germany, [2]Axiogenesis AG, Cologne, Germany.

The use of cardiac myocytes is becoming increasingly important for drug safety testing. Unique features of certain planar patch clamp workstations, coupled with ease-of-use and higher data throughput, make these devices ideal tools for ion channel screening and safety testing. Using stem cell derived cardiac myocytes, recordings could be made not only in the voltage-clamp mode but also in the current-clamp mode on a planar patch clamp workstation. This demonstrates for the first time parallel current-clamp recordings on a planar patch clamp workstation. Ion channels important in drug discovery, such as hERG and voltage-gated Na+, Ca2+ and K+ channels in the voltage-clamp mode from stem cell derived cardiac myocytes will be shown. In addition, action potential recordings in the current-clamp mode at 35°C, and modulation of the action potentials by hERG active compounds, will also be shown.



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***In Silico* Studies of Cardiac Inotropy using a New Model of Force Generation**

Jose L. Puglisi[1], Jorge A. Negroni[2], Donald M. Bers[1].
[1]University of California, Davis, Davis, CA, USA, [2]Universidad Favaloro, Buenos Aires, Argentina.

An improved model of force generation was incorporated into a complete mathematical description of action potential (AP), ionic currents and Ca^{2+} transient of the rabbit ventricular myocyte (LabHEART 5.0). This new model reproduces the main events involved in Excitation-Contraction Coupling, namely the AP (excitation), the shortening (contraction) and the Ca^{2+} transient as the link between them. LabHEART 5.0 was able to reproduce isotonic and isometric contractions and the classical curves of Force vs. Ca^{2+} and Force vs cell length. Cardiac inotropy was investigated by simulating the application of isoproterenol (ISO). This effect was achieved by altering L-type Ca^{2+} current, the slowly activating delayed rectifier K^+ current, sarcoplasmic reticulum (SR) Ca^{2+} pump, SR Ca^{2+} leak, myofilament Ca-sensitivity and cross-bridge cycling. The latter modification was essential for replicating the ISO-induced increase in force generation/shortening development experimentally observed. AP duration (APD, for 90% of repolarization) adaptation to pacing frequencies was also examined. ISO shortened APD at all frequencies with respect to control and flattened the adaptation curve, thus allowing an APD compatible with short cycle length (up to 5 Hz). ISO also increased the Ca^{2+} transient dynamic range by keeping a low diastolic level while increasing the peak Ca^{2+} at all the simulated frequencies (0.5 to 9 Hz). This model provides a useful framework to study cardiac inotropy and constitutes a starting point to investigate the electromechanical feedback in cardiac performance. The new version LabHEART 5.0 is freely available online at www.labheart.org.

Voltage-gated Ca Channels I

2665-Pos

Monte Carlo Simulation of Free Energy Components: Energetics of Selective Binding in a Reduced Model of L-Type Ca Channels

Janhavi Giri[1,2], Bob Eisenberg[2], Dirk Gillespie[2], Douglas Henderson[3], Dezső Boda[4].
[1]University of Illinois at Chicago, Chicago, IL, USA, [2]Rush University Medical Center, Chicago, IL, USA, [3]Brigham Young University, Provo, UT, USA, [4]University of Pannonia, Veszprém, Hungary.

A reduced model of voltage-gated L-type Ca channels is used to study the energetics of selective binding of Ca^{2+} versus monovalent and divalent cations. Widom's particle insertion method is combined with Grand Canonical Monte Carlo simulations to compute the electrostatic and excluded volume components of the free energy difference between channel and bath. We have shown (in ~ 30 papers) that selectivity of the L-type Ca channel and voltage activated