930-Pos Board B809

Ca^{2+}- and Thromboxane-Dependent Distribution of Functional MaxiK Channels in Cultured Astrocytes

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Large-conductance, voltage- and Ca^{2+}-activated K^+ channels (MaxiK, BK) are broadly expressed ion channels typically observed as a plasma membrane protein in various cell types. In murine astrocyte primary cultures, which are more indicative of *in-vivo* reactive astrocytes rather than resting astrocytes, our previous results using high-resolution confocal microscopy have revealed the novel finding that MaxiK pore-forming α subunit (MaxiKα) is distributed intracellularly, colocalized along the microtubule network. This MaxiKα association with microtubules was further confirmed by *in vitro* His-tag pulldown assays, co-immunoprecipitation assays from brain lysates, and microtubule depolymerization experiments. Changes in intracellular Ca^{2+} elicited by general pharmacological agents, caffeine (20mM) or thapsigargin (1μM), resulted in increased MaxiKα labeling at the plasma membrane. More notably U46619, a stable analog of thromboxane A2 (TXA2) which triggers Ca^{2+}-release pathways and whose levels increase during cerebral hemorrhage/trauma, also elicits a similar increase in MaxiKα surface labeling. We now show using whole-cell patch clamp recordings that U46619 stimulated cells develop a ~3-fold increase in current amplitude. This data indicates that TXA2 stimulation results in the recruitment of additional, functional MaxiK channels to the surface membrane. These changes in MaxiKα plasma membrane distribution are effectively blocked by preincubating astrocytes with a cell permeable Ca^{2+}-chelator, BAPTA-AM, or by microtubule disruption prior to stimulation. While microtubules are largely absent in mature astrocytes, our immunohistochemistry results in brain slices show that cortical astrocytes in the developing newborn mouse brain (P1) have a robust expression of microtubules that significantly colocalize with MaxiKα. The results of this study provide the novel insight that suggests Ca^{2+} released from intracellular stores, may play a key role in regulating the traffic of intracellular, microtubule-associated MaxiKα stores to the plasma membrane of reactive astrocytes. Supported by NIH.

Voltage-gated Ca Channels I

931-Pos Board B810

Membrane Voltage More Efficient In Closing Than Opening $Ca_V1.2$?

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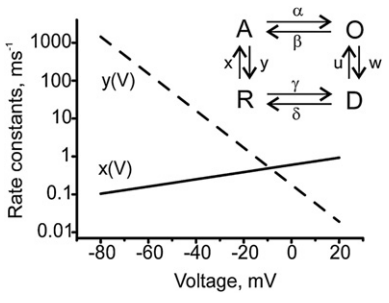
Point mutations in a gating-related sequence stretch in the pore-lining segment IIS6 of $Ca_V1.2$ (779-782:LAIA motive) convert the high-voltage activated calcium channel into a low-voltage activated one. Here we analyze the changes in current activation and deactivation induced by these and glycine mutations in this region. Our model describes channel activation as voltage-dependent sensor movement and a voltage-independent pore opening and deactivation as voltage-dependent return of the sensor and subsequent pore closure.

An inverse problem approach enabled the estimation of current activation and deactivation rate constants from 16 mutants and wild type $Ca_V1.2$ with narrow confidence intervals. Current activation, deactivation and steady-state activation of wild type and 12 mutants could be fitted with identical voltage dependencies of the voltage sensing machinery (x(V),y(V)). Steeper voltage dependence of y(V) compared to x(V) (see Figure) suggest that a membrane hyperpolarisation more efficiently closes than a depolarization opens the channel. Mutations in IIS6 of $Ca_V1.2$ destabilizing the closed state simultaneously appear to stabilize the open state in all 16 mutants.



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932-Pos Board B811

Position Specificity of the Glycine Residues in IS6 of the L-type Cav1.2 Channel

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In the Timothy Syndrome (LQT8), mutations of the distal glycine residues in IS6 severely delayed the inactivation kinetics of Cav1.2 (Splawski et al., 2004, 2005). Our mutational analysis confirmed that Ala mutations of any glycine within IS6 significantly decreased the inactivation kinetics (Raybaud et al., 2006). To evaluate the position-specificity of these residues in IS6, we performed a glycine scan of this region between S423 and E437 (NLVLGVLSGE). We hypothesized that introduction of a glycine residue at neighboring positions within the LTQ8 mutants might rescue the Cav1.2 channel inactivation kinetics. Although S423G, F424G, and L434G yielded channels with slightly faster inactivation kinetics than the wild-type Cav1.2 channel, the double mutants G422A/S423G and G422A/F424G failed to rescue the channel normal inactivation kinetics. As G422 is located next to a bulky phenylalanine residue, we aimed to evaluate the role of steric hindrance in controlling channel inactivation kinetics. F421G, F421A, F421W, as well as the F421G/G422A double mutant have been characterized. Both F421A and F421G behaved like the wild-type channel whereas F421W did not yield functional currents. The double mutant F421G/G422F that switched the positions of the wild-type Phe and Gly residues inactivated like the G422A mutant. Furthermore, permutations such as F421A/G422F and F421A/G422A also yielded the G422A phenotype. Altogether, these experiments confirm the position-specificity of the 3 glycine residues in IS6 in modulating the inactivation kinetics of Cav1.2. In contrast, none of our mutations altered the voltage-dependence of activation suggesting that residues in IS6 do not play a determinant role in the activation properties of Cav1.2. *Supported by the Heart and Stroke of Canada and the Canadian Institutes of Health Research.*

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Distinctive Inactivation Profiles of $Ca_V1.2$ Channels Encoding Different Timothy Syndrome Mutations in Various Alternative Splicing Backgrounds

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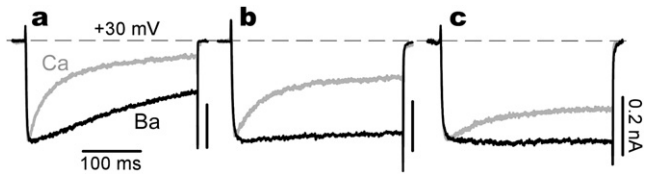
Timothy Syndrome is a $Ca_V1.2$ channelopathy, wherein mutations in the S6 segment of domain II affect channel inactivation. Interestingly, different mutations (G402S or G406R) within alternatively spliced exons (8a or 8) entail characteristic disease phenotypes, including autism, syndactyly, and long QT syndrome. Investigating Timothy Syndrome thereby promises mechanistic traction into these complex outcomes. The prevalence and distribution of channels bearing exon 8 versus 8a may explain some phenotypic variation. Here, we examine such variation at a more fundamental level, resolving intrinsic differences of inactivation among distinct mutant channels. Specifically, $Ca_V1.2$ inactivation comprises two separate mechanisms, voltage-dependent inactivation (VDI), and Ca^{2+}/calmodulin-mediated inactivation (CDI) (Barrett & Tsien PNAS 2008). Systematic mutagenesis of S6 domains in $Ca_V1.3$ suggest that VDI and CDI alterations can be dissociated, and changes in channel activation are likely (Tadross *et al*, this meeting). Our data here furnish remarkable examples of differing CDI/VDI effects (**a**, wild-type, with Ba^{2+} current decay showing VDI, and Ca^{2+} decay showing CDI; **b**, **c**, distinct CDI/VDI alterations). It would be interesting if distinctive disease phenotypes and therapeutics ultimately correspond to specified deficits of VDI, CDI, or both.



934-Pos Board B813

Silencing of Cav1.2 gene in Rat Neonatal Cardiomyocytes by Lentiviral delivered shRNA

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Background: Two types of L-type Ca Channels are expressed in the heart: Cav1.2 (α_{1C}) and Cav1.3 (α_{1D}). In contrast to α_{1C}, α_{1D} Ca channel is highly expressed in the sinoatrial node and atria, and is involved in the impulse generation and propagation through the AV node. Deletion of the α_{1C} gene results in embryonic lethality before E14.5 and there are no pharmacological or biophysical means to separate α_{1D} from α_{1C} Ca currents. The aim of this study

was to establish a model to characterize α_{1D} Ca current using the novel technology of RNA interference.
Methods and Results: Rat neonatal cardiomyocytes (RNC) were transfected with α_{1C} specific siRNA using lipofectamine which resulted in 50% silencing at the mRNA/protein level. Limited by the low transfection effieciency (50%) in the RNC, we cotransfected α_{1C} siRNA with cy3 labeled Human GAPDH siRNA and sorted out the fluorescent cells using FACS to separate tranfected and non-transfected cells. The level of silencing of α_{1C} in enriched transfected cells reached only 65% efficiency. To achieve higher transfection efficiency, we generated and infected RNC with a lentivirus construct carrying the α_{1C} siRNA sequence under a U6 promoter. Using this model, we achieved 100% transfection efficiency, and more than 90% silencing of the α_{1C} gene confirmed by real-time PCR, Western blot, and immunoflourecence. These biochemical results were confirmed electrophysiologically by measurements of total L-type Ca current which was reduced by 80% in transfected cells.
Conclusion: Lentiviral shRNA is an efficient model for post-transcriptional gene silencing of ion channels in primary cardiomyocytes. This novel approach provides a valuable mean for assessing the differential roles of α_{1C} and α_{1D} Ca channels in native cardiomyocytes and could be used to examine their roles in physiological and pathological settings.

935-Pos Board B814

Construction Of Functional N-type Ca2+ Channels (Cav2.2) With Accessible External Epitope Tags Suitable For Live Cell Labeling

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Recent studies of voltage-gated Ca^{2+} channels indicate that mechanisms other than alterations in gating contribute to modulation. For example, internalization of receptor/channel complexes (e.g., ORL1) or formation of non-conducting species (e.g., RGK proteins), potentially contribute to $Ca_V2.2$ function. An essential technique for investigating these phenomena is the ability to specifically label fully functional $Ca_V2.2$ α-subunits (*CACNA1B*) in the plasma membrane of living cells. Towards this end, we attempted a systematic insertion of a hemagglutinin (HA) epitope tag into each of the 12 predicted extracellular loops of the rat $Ca_V2.2$ α-subunit. The first round of mutagenesis resulted in the addition of sites to 1E1 (domain 1, extracellular loop 1), 1E2, 2E2, 3E1, 3E2 and 4E3. A sequence coding for a short flexible linker plus two contiguous copies of the hemagglutinin epitope was ligated into each site. The resulting constructs were electroporated into HEK293 cells along with plasmids encoding Ca^{2+} channel β_{2a} and $\alpha_2\delta$ subunits, and the K^+ channel IRK1. Robust ''rim type'' immunofluorescent labeling (in living cells) was detected for 4 of 6 clones (1E2, 3E1, 3E2 and 4E3). Expression of these constructs in HEK293 cells produced channels with electrophysiological properties similar to wildtype as determined by whole-cell patch-clamp with 10 mM Ca^{2+} as the charge carrier. Average peak currents were (in nA) -5.4 ± 0.7 for the control and -3.8 ± 0.7, -1.2 ± 0.2, -3.1 ± 0.7 and -4.0 ± 0.7 for 1E2, 3E1, 3E2 and 4E3, respectively. The IV curves and individual current trajectories for each clone were superficially similar to the wildtype. These clones should provide powerful tools for the study of trafficking and modulatory mechanisms of $Ca_V2.2$. Additionally, the tagging strategy may be applicable to additional members of the Ca_V and Na_V families of voltage-gated channels.

936-Pos Board B815

Role Of Gamma Subunit In The Targeting Of Functional Cardiac L-Type Ca^{2+} Channels

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The cardiac isoform γ6 of the Ca^{2+} channel γ subunit family has been proposed to inhibit T-type Ca^{2+} channels by reducing the channel's availability for activation (Hansen et al., 2004, J Mol Cell Cardiol). Here we investigated how the γ6 subunit (long transcript) regulates L-type Ca^{2+} channels expressed in tsA201 cells.
First, the sub-cellular localization of the N-terminally GFP-tagged γ6 was visualized by confocal imaging. Expressed alone or with any combination of other types of subunits, the γ6 targeted well to the plasma membrane.
Second, when the γ6 subunit (long transcript) was co-expressed with α1C and β2a subunits, it dramatically reduced the density of ionic currents recorded with 10 mM Ba^{2+} from 142 ± 26 pA/pF (n=4) in control to 3 ± 2 pA/pF (n=7). The gating charge was also nearly eliminated in the presence of the γ6 subunit (from 47 ± 12 fC/pF in control to less than 5 fC/pF in cells with the γ6). The reduction in current and gating charge was independent on GFP-tagging of the γ6.
Taken together, these results indicate that the cardiac γ6 subunit regulates expression/functional targeting of cardiac L-type Ca^{2+} channels.

937-Pos Board B816

Calreticulin Negatively Regulates the Surface Expression of α1D L-Type Calcium Channel

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Background: Quality control of several proteins is strictly regulated by molecular chaperones in the endoplasmic reticulum (ER). Calreticulin, an ER Ca2+ binding chaperone, has been shown to regulate the surface expression of several membrane proteins including the cyctic fibrosis transmembrane conductance regulator (CFTR) which under oxidative stress results in its internalization and proteasomal degredation. Decrease of L-type Ca current and channel protein has been described in autoimmune associated congenital heart block. Here, we demonstrated a novel mechanism of down-regulation of α_{1D} L-type Ca channel using native human fetal cardiac cells and tsA201 cell line.
Methods and Results: Using Confocal microscopy, we found surface staining of calreticulin on cultured human fetal cardiomyocytes (HFC) gestational age 18-24 weeks. Coimmunoprecipitation from HFC using anti-α_{1D} antibody, and probing with anti-calreticulin antibody revealed a 46 kDa band corresponding to calreticulin. Overexpressing calreticulin in human embryonic kidney cells (tsA201) resulted in a decrease in surface expression of α_{1D} L-type Ca Channel. Electrophysiological studies showed that co-transfection of calreticulin with α_{1D} L-type Ca Channel led to 55% inhibition of the α_{1D} Ca current expressed in tsA201 cells.
Conclusions: These results show the first evidence that calreticulin: 1) is found on the cell surface of human fetal cardiomyocytes; 2) is coimmunoprecipitated with α_{1D} L-type Ca Channel; 3) negatively regulates α_{1D} surface expression; 4) decreases α_{1D} Ca current in tsA201 cells co-expressed with α_{1D} and calreticulin. The data demonstrated a novel mechanism of modulation of α_{1D} Ca channel, which may be involved in numerous pathological settings such as congenital heart block.

938-Pos Board B817

Inhibition Of Recycling Endosomes By Brefeldin-A Prevents KLHL1-mediated Upregulation Of α1h T-type Currents

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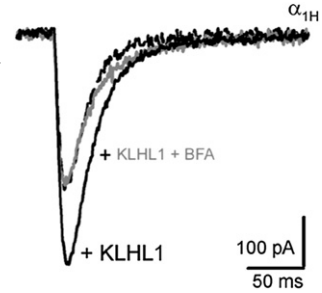
The Kelch-like 1 (KLHL1) actin binding protein interacts with α_{1H} calcium channels and increases calcium current density *via* interaction with the actin cytoskeleton, resulting in an increase in the number of channels at the membrane. We probed the effect of Brefeldin A (BFA), which affects protein transport *via* disruption of the trans-Golgi network and by blocking vesicle recycling. HEK 293 cells stably transfected with α_{1H} were incubated with various BFA concentrations; overnight incubations resulted in the elimination of basal α_{1H} currents (IC_{50}=21μM), consistent with impaired channel trafficking from the Golgi complex. Interestingly, low [BFA] (107 nM) and/or short-term incubations (3.6 μM for 1 hr) did not alter endogenous α_{1H} levels, yet completely eliminated the KLHL1 effect. Current deactivation kinetics changes originally observed in the presence of KLHL1 persisted in the presence of BFA, indicating that BFA does not affect the direct interaction of KLHL1 with α_{1H} already present at the plasma membrane.



Our data suggests KLHL1 up-regulates channel number by increasing α_{1H} re-insertion tino the membrane *via* recycling endosomes, a process that involves stabilization of the actin cytoskeleton. Supported by AHA-0615508Z (KA) and NSF- 0641141 (EPR).

939-Pos Board B818

Cardiac L-type Ca Channel as an Oxygen Sensor; Possible Involvement of Ca/Calmodulin Binding Domain

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Cellular oxygen sensing is defined as the ability of a cell to respond to changes in extracellular oxygen pressure through regulation of membrane ionic currents, mitochondrial oxidative phosphorylation or transcription of regulatory proteins. In the heart hypoxia has been shown to alter ionic currents through mitchondrial redox regulation and phosphorylation by kinases. Here we report