2675-Pos

Distinctive Inactivation Defects of Differing Mutant Calcium Channels Underlying Timothy Syndrome

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Timothy Syndrome (TS) is a multisystem disorder characterized by autism, immune deficiencies, and cardiac arrhythmias. Intriguingly, the underlying defect comes down to a single point mutation (either G402S or G406R) in the IS6 region of $Ca_V1.2$ channels. These channels are critical conduits of Ca^{2+} entry into the heart, smooth muscle and brain. As such, these channels employ two forms of feedback regulation—voltage-dependent inactivation (VDI) and Ca^{2+}/calmodulin-dependent inactivation (CDI). In TS, these regulatory mechanisms are disrupted, resulting in inappropriate Ca^{2+} feedback. Given that the pattern of multisystem pathology differs for the two types of mutant channels, we here undertook in-depth biophysical analysis of the altered inactivation in each of these constructs. As reported, both mutants exhibited strongly attenuated VDI. Rather surprisingly, however, both constructs also demonstrated a clear reduction of CDI, in contrast to a previous study reporting selective weakening of VDI (*PNAS***105**:11987). Further analysis revealed that the CDI deficits in the two mutants may arise from very different mechanisms. For G406R, voltage-dependent activation is strongly shifted to more negative potentials, while estimated maximal open probability ($P_{O/max}$) at saturating depolarization was only slightly altered. According to an allosteric mechanism of CDI (*Biophys J* **96**:222a), this favoring of channel activation would reduce CDI, because opening would be enhanced even within inactivated channels (i.e., the current decrease seen upon channel inactivation would be lessened). By contrast, the G402S mutation caused a marked depolarizing shift in voltage-dependent activation, with largely unchanged $P_{O/max}$. This outcome would sharply diminish channel opening at physiological voltages, yielding attenuated CDI via decreased entry into inactivated states. Recognizing these divergent mechanisms of CDI disruption may shed light on the differing disease phenotypes elaborated by the two mutations, and ultimately prove beneficial in tailoring treatments for each TS population.

2676-Pos

Diminished Dihydropyridine Block of Timothy Syndrome Cav1.2 Channels Independent from Mutation-Altered Open State Inactivation

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Timothy syndrome (TS) is a multisystem developmental disorder presenting clinical phenotypes of autism and cardiac arrhythmias. The disease is linked to single amino acid mutations (G402S or G406R) in the Cav1.2 L-type calcium channel which dramatically disrupt voltage-gated inactivation from the open state, as seen in electrophysiological recordings with barium as the charge carrier to remove calcium-dependent inactivation. Initial reports suggested that inactivation-deficient TS channels are less sensitive to DHP antagonists, presumably because these drugs preferentially inhibit inactivated channels. Here we thoroughly investigated inactivation and isradipine inhibition of G406R channels at voltages where the channel inactivates directly from closed states. Interestingly, despite dramatic differences in open state inactivation, closed state inactivation during long 25s conditioning pulses is minimal and not distinguishable between WT and G406R channels. Nevertheless, TS channels are still less sensitive than WT channels to DHP block at these voltages: 10nM isradipine blocks 10% of G406R channels vs. 30% of WT channels at −100mV and 35% of G406R channels vs. 70% of WT channels at −40mV. Investigation of −100mV block by multiple concentrations of isradipine revealed that G406R channels in deep closed states have a greatly reduced affinity (Kd ~4.5 greater) for isradipine. To test how altered −100mV block affects block at modestly depolarized potentials, a drug concentration was chosen for TS channels (50nM isradipine) that produced the same 30% block at −100mV. Strikingly, when the change in affinity at −100mV was accounted for, block at −40mV prior to channel opening was identical between mutant and WT. These results suggest that the G406R mutation alters rested state block and that this accounts for the reduced drug block at modestly depolarized voltages, independent of the severe disruption in open state inactivation of the channel.

2677-Pos

Rare Missense Mutations in the Calcium Channel β2 Subunit of Autistic Patients

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Calcium channels are crucially involved in brain development and neuronal function. Mutations in the pore-forming Cav subunits of high- and low-voltage dependent calcium channels (VDCC) have been found in patients with autistic syndromes (Splawski et al., Cell 2004;119:19-31; Splawski et al., PNAS 2005;102:8089-96; Splawski et al., JBC 2006;281:22085-91). In Timothy syndrome, the G406R mutation of Cav1.2 results in a reduction of inactivation rate. Such biophysical effects can likewise be induced by the influence of auxiliary VDCC β subunits. For instance, we demonstrated a novel mechanism of β subunit modulation: the inactivation of VDCC is under length-dependent control of the β2 subunit N terminus (Herzig et al., FASEB J 2007;21:1527-38). A similar mechanism operates with the β1 subunit (Jangsangthong et al., Pflugers Arch 2009, in press).

Therefore, the β2-subunit gene was screened for mutations in 155 patients with Autistic Spectrum Disorder (ASD). We detected several new variations and compared the genotypes with 375 matching controls. (Male to female ratio for both groups is 1:4.). Statistical analysis of preselected variations showed two significant SNPs in functional intronic regions (χ^2 p = $5x10^{-6}$ and χ^2 p = $9x10^{-3}$).

Furthermore, we also identified several rare ASD-specific missense mutations at the gene locus of the β2 subunit. These mutations occur in highly conserved domains and may lead to alterations in the β2 subunit function, e.g. by interfering with subunit phosphorylation. The affected amino acids are highly conserved among species, suggesting an importance for topology and function of the subunit. We will clone these variations into expression vectors and characterize their functional effects by electrophysiological studies. These studies may provide new insights into molecular mechanisms leading to ASD.

2678-Pos

Effect of Pregabalin on Synaptic Transmission in Rat Dorsal Horn Ganglion and Dorsal Horn Co-Cultures

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The calcium channel alpha-2-delta (α2δ) subunit is an auxiliary subunit associated with voltage-dependent calcium channels, and is implicated in the trafficking and functional expression of the calcium channel complex.

Upregulation of the α2δ-1 subunit in rat dorsal root ganglion (DRG) neurons occurs in several animal models of neuropathic pain, and results in an increase in trafficking of α2δ-1 to the presynaptic terminals of DRG neurons. This is inhibited by the α2δ-1 ligand pregabalin (Bauer et al., 2009).

Co-cultures of embryonic rat spinal cord neurons and dorsal root ganglion neurons were used to examine synaptic transmission between the neurons, and the impact of pregabalin on this process. We have examined synaptic transmission by using both Fura-2 calcium imaging and *in vitro* electrophysiology.

The $F_{340/380}$ ratio was increased in DRGs upon exposure to capsaicin because of Ca^{2+} entry, and (with a delay) there was also an elevation in dorsal horn neurons. In a parallel experiment, the corresponding increase in observed EPSCs frequency in dorsal horn neurons was 3.0 ± 0.5-fold (n=14). The non-NMDA receptor antagonist CNQX (10 μM) caused a complete and reversible inhibition of the observed EPSCs.

In dorsal horn monocultures there was no significant increase in EPSC frequency in response to capsaicin application. In addition, no increase in the $F_{340/380}$ ratio was observed in dorsal horn neuron monocultures in response to capsaicin.

Co-cultures were also incubated with pregabalin. The observed increase in EPSC frequency in control cells was 3.2 ± 1.5-fold (n=9), compared to a 1.5 ± 0.3-fold (n=6) increase cells treated with pregabalin (100 μM) for 48 hours. These data suggest that chronic pregabalin treatment reduced synaptic transmission between DRGs and dorsal horn neurons.

Bauer et al. (2009). J Neurosci. Apr 1;29(13):4076-88.

2679-Pos

The Cardiac α_{1c} Subunit is Down Regulated by Pharmacological Preconditioning

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Background and Purpose: It is well established that Pharmacological Preconditioning (PP), achieved with openers of mitochondrial K_{ATP} channels like diazoxide, leads to cardioprotection against subsequent ischemia. However, the changes in Ca^{2+} homeostasis during PP are poorly understood. Here, we investigate the effects of PP on the L-type Ca^{2+} channel of the adult heart.

Experimental approach: Preincubation with diazoxide (100μM) for 90 min was used to induce PP in two preparations: Isolated hearts from rat Wistar and enzymatically dissociated rat ventricular myocytes. Cardiomyocytes were voltage-clamped to measure L-type Ca^{2+} currents (I_{Ca}) with the whole-

cell patch-clamp technique and Ca^{2+} signals and ROS production were measured with the fluorescent probes, Fluo 3-AM and CM-H_2DCFDA, respectively. The levels of the α_{1c} subunit, obtained from diazoxide preconditioned hearts, were measured in the membrane fraction of rat ventricles by Western blot. The ROS scavenger NAC was used to examine the role of ROS on the L-type Ca^{2+} channel after PP in both preparations.
Results: Diazoxide induced PP was accompanied by a significant downregulation of the α_{1c} subunit in the membrane fraction and by a reversible reduction in the amplitude of I_{Ca} and Ca^{2+} transients. These effects were complete within 90min and were prevented by NAC. Diazoxide significantly increased ROS production in cardiomyocytes. The reduction of I_{Ca} and Ca^{2+} transients by PP were prevented by the mitochondrial K_{ATP} channel blocker 5-HD.
Conclusions: Pharmacological preconditioning induced with diazoxide, leads to downregulation of the α_{1c} subunit of the L-type Ca^{2+} channel. This reduces the influx of Ca^{2+} through these channels and may contribute to attenuate the overload of Ca^{2+} during reperfusion.
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2680-Pos
Voltage-Dependent Kappa Opioid Modulation of Calcium Currents Elicited by Action Potential Waveforms in Neurohypophysial Terminals
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Release of neurotransmitter is activated by the influx of calcium. Inhibition of Ca^{2+} channels results in less calcium influx into the terminal and, presumably, a reduction in transmitter release. In the nerve terminals of the neurohypophysis, voltage-gated calcium channels (VGCC) are primarily controlled by membrane voltage and their activity can be modulated, in a voltage-dependent manner, by their interaction with G-protein subunits. Endogenous opioids also affect (inhibit) these calcium channels, upon binding to μ- and κ-receptors at the terminals.
Voltage-dependent relief of G-protein inhibition of VGCC is achieved with either a depolarizing square pre-pulse or by action potential waveforms. Both protocols were tested in the presence and absence of opioid agonists targeting the μ- and κ-receptors. The κ-opioid VGCC inhibition is relieved by such pre-pulses, suggesting that this receptor is involved in a voltage-dependent membrane-delimited G-protein pathway. In contrast, μ-opioid inhibition of VGCC is not relieved by such pre-pulses, indicating a voltage-independent diffusible second-messenger signaling pathway. Furthermore, κ-opioid inhibition is also relieved during stimulation with action potential bursts with physiological characteristics. This indicates the possibility of activity-dependent modulation *in vivo*.
Differences in the facilitation of Ca^{2+} channels due to specific G-protein modulation during a burst of action potentials may contribute to the fine-tuning of Ca^{2+}-dependent neuropeptide release in other central nervous system synapses, as well. [Supported by NIH Grant NS29470].

2681-Pos
Selective Inhibition of T-Type Calcium Channels by Endogenous Lipoamino Acids
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T-type calcium channels, i.e. Cav3.1, Cav3.2 and Cav3.3 channels, have important roles in cell excitability and calcium signalling and contribute to a wide variety of physiological functions especially in nervous system. Over the past few years, several endogenous ligands regulating Cav3 activity were identified, including bioactive lipids such as the endocannabinoid anandamide (N-arachidonoyl ethanolamine). We now provide evidence that the T-type / Cav3 calcium channels are potently and reversibly inhibited by various lipoamino acids, including N-arachidonoyl glycine (NAGly, IC50 ~ 600 nM for Cav3.2) and N-arachidonoyl 3-OH-gamma-aminobutyric acid (NAGABA-OH, IC50 ~200 nM for Cav3.2). This inhibition involves a large shift in the Cav3.2 steady-state inactivation and persists during fatty acid amide hydrolase (FAAH) inhibition as well as in cell-free outside-out patch. It appears that lipoamino acids are the most active endogenous ligand family acting on T-channels. Importantly, lipoamino acids have weak effects on high-voltage-activated (HVA) Cav1.2 and Cav2.2 calcium currents, on Nav1.7 and Nav1.8 sodium currents as well as on TRPV1 and TASK1 currents. These data indicate that lipoamino acid effects may be selective of T-type channels over HVA calcium channels, sodium channels as well as the anandamide-sensitive TRPV1 and TASK1 channels. It also suggests that these ligands can modulate multiple cell functions via T-type calcium channel regulation. In line with this, we found that lipoamino acids evoke a thermal analgesia in wild-type but not in Cav3.2 KO mice. Collectively, our data identify lipoamino acids as a new potent and selective family of endogenous T-type channel inhibitors.

2682-Pos
Chronic Alcohol Consumption Blunts β-Adrenergic Responsiveness in Left Ventricular Cardiomyocytes
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Alcoholic cardiomyopathy (ACM) develops from long-term, excessive consumption of alcohol. Initially, ACM is asymptomatic but continued alcohol abuse leads to reductions in cardiac contractility, the onset of arrhythmias, chamber dilation and congestive heart failure. This study was carried out to examine the effects of chronic alcohol on basal and β-adrenergic-stimulated properties of Ca^{2+} transients during excitation-contraction (E-C) coupling. Rats were pair-fed DeCarli and Lieber control and alcohol liquid diets for 120 days prior to isolating left ventricular myocytes. Under basal conditions, there was no change in the amplitude of electrically-triggered $[Ca^{2+}]_i$ transients (Control, 296 ± 21nM vs. Alcoholic, 260 ± 18 nM) or contraction (Control, 11.9 ± 0.6 μm, Alcoholic,12.7 ± 1.2 μm. However, a blunted inotropic response (increase over basal: Control, 90 ± 19% vs. Alcoholic 39 ± 10 %) was observed in the presence of submaximal isoproterenol stimulation. In addition, maximal isoproterenol and forskolin stimulation do not improve the inotropic response of the alcoholic myocytes, suggesting a functional impairment in the initial Ca^{2+} release steps of E-C coupling. Consistent with the reduced $[Ca^{2+}]_i$ transient amplitude, the Ca^{2+} current ($I_{Ca,L}$) responses to isoproterenol were also markedly reduced in cardiomyocytes from alcohol-fed animals. Surprisingly, measurement of L-type calcium channel expression by dihydropyridine (DHP) binding and real-time PCR, revealed an increased number of DHP binding sites (Control $B_{max} = 197 \pm 60$ fmol/mg vs. Alcoholic $B_{max} = 335 \pm 45$ fmol/mg, $P < 0.05$) and α1C subunit expression (Alcoholic $2^{-\Delta\Delta Ct}1.69 \pm 0.03$, Control $2^{-\Delta\Delta Ct}0.96 \pm 0.02$, $P < 0.0005$), respectively. This loss of L-type calcium channel activity, accompanied by an increased channel expression with chronic alcohol consumption may be a precipitating factor in alcoholic heart disease, leading to the onset of other adaptive mechanisms and, eventually, the clinical syndrome of heart failure.

2683-Pos
Characterization of the Calmodulin-Binding Site in the N Terminus of $Ca_V1.2$
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$Ca_V1.2$ is an L-type Ca^{2+} channel from a family of voltage dependent Ca^{2+} channels(VDCC)distributed mainly in cardiac and smooth muscle, endocrine cells and neurons, which produce calcium influx in response to membrane depolarization. Interaction of calmodulin (CaM) with the C-terminus (CT) of the L-type $Ca_V1.2$ channel is crucial for Ca^{2+}-dependent inactivation (CDI). CaM also binds to the N-terminus (NT), and a CaM-formed "bridge" between CT and NT has been proposed to control CDI.
We characterized the interaction of CaM with its NT-binding peptide. Using ITC, we determined the binding of CaM to the NT-binding site is Ca^{2+}-dependent with an affinity of 0.6 μM. The Ca^{2+} dependence of the NT-CaM interaction makes it a plausible candidate for a reversible, Ca^{2+}/CaM-dependent regulatory process such as CDI. However, our results do not support a model in which CaM forms a direct "bridge" between the N and C-terminal CaM binding sites. NSCaTE (N-terminal spatial Ca^{2+} transforming element), which appears to play a substantial role in CDI of CaV1.3, does not appear to be strongly involved in the inactivation process in $Ca_V1.2$. Mutations in NT of $Ca_V1.2$ that abolished the binding of CaM only slightly weakened the CDI but also accelerated the VDI. CaM did not foster an interaction between the CaM-binding peptides of NT and CT. Thus, the role of CaM's interaction with the $Ca_V1.2$ NT remains to be determined.

2684-Pos
The L-Type Calcium Channel C-Terminus is a Mobile Domain that Competes with Calmodulin Modulation of Calcium Current
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The L-type Ca channel (CaV1.2) distal carboxyl-terminus (CCt) has multiple functions. CCt inhibits L-type calcium current (ICa,L), and is a mobile element that translocates to the nucleus where it regulates CaV1.2 transcription. CCt