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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.

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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.

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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.

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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.

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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-