The Cardiac L-Type Calcium Channel Distal Carboxyl Terminus is a Reverse use Dependent Inhibitor of Ca Current in Cardiomyocytes

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and YFP-tagged carboxy-terminal peptides of $Ca_V1.3$, $Ca_V2.1$, $Na_V1.4$, and $Na_V1.5$ channels. Remarkably, the maximal FRET ratios, computed via 3^3-FRET and E-FRET methods, equaled unity, arguing well for a 1:1 stoichiometry of apoCaM binding across Ca_V and Na_V ion-channel families. These results mirror functional findings that a single CaM, pre-associated to the carboxy-termini of these channels, suffices for robust modulation of channel gating. More broadly, these experiments underscore the power of FRET 2-hybrid assay to discern multiple aspects of binding interactions within a live-cell context.

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Resolving the Grip of the Distal Carboxy Tail on the Proximal Calmodulatory Region of Ca_V Channels

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Ca^{2+}/calmodulin-dependent inactivation (CDI) of Ca_V channels plays a crucial role in the homeostasis of intracellular Ca^{2+}, and serves as an ideal prototype for investigating Ca^{2+} feedback regulation within biological systems. The prevailing view of CDI mechanism is that Ca^{2+}-free calmodulin (apoCaM) preassociates with the IQ domain of the proximal carboxy terminus of channels, and this 'resident' calmodulin (CaM) acts as Ca^{2+} sensor to somehow trigger CDI upon Ca^{2+} binding. The downstream CaM/channel configurations leading to CDI are complex and under investigation at present. Contrasting with this complexity, $Ca_V1.3$ and $Ca_V1.4$ feature a long-carboxy-tail splice variant that minimizes CDI by a beautifully simple mechanism. We recently combined electrophysiology and a genetically encoded fluorescent sensor to record CDI and CaM concentration simultaneously (*Nature***463**:968), thereby demonstrating that the distal-carboxy-tail (DCT) harbors an ICDI module that competes with apoCaM for binding to the IQ domain. By kicking off apoCaM, ICDI diminishes CDI in an eminently straightforward manner; thus, the IQ/ICDI complex now looms as a simplified prototype for novel channel modulators, and as an easy entrez into complex CaM/channel configurations underlying CDI. Here, we perform alanine-scanning mutagenesis of the entire IQ domain and ICDI domain. Hotspot mutations which reduce IQ/ICDI binding affinity also commensurately diminish ICDI function (i.e., permit restoration of CDI). Furthermore, the ensemble of effects can be well fit with a Michaelis-Menten equation incorporating the presence of a competitive inhibitor. Thus, the collection of hotspots can be used to identify the minimal functional segments of IQ/ICDI interaction to facilitate structure building, and to provide clues for designing small peptides and/or molecules that regulate CDI of specific Ca_V channels.

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Background. The LTCC distal C-terminus (DCT) is proteolytically cleaved, and re-associates with the LTCC complex to regulate calcium channel function. DCT reduces LTCC barium current (IBa,L) in reconstituted channel complexes and mediates the fight or flight response in DCT knockout mice; yet the contribution of DCT to ICa,L in native systems is unexplored. This study examines the beat-to beat contribution of DCT to LTCC calcium homeostasis in native cardiomyocytes.

Methods. We measured LTCC current with DCT co-expressed in cardiomyocytes. We also heterologously co-expressed DCT and CaV1.2 constructs with a stop corresponding to the predicted proteolytic cleavage site, Cav1.2Δ1801, and a shorter deletion corresponding to well-studied construct, Cav1.2Δ1733 along with CaVβ2 subunits.

Results. DCT was found to inhibit IBa,L, but not ICa,L in cardiomyocytes. DCT blocked IBa,L carried by both Cav1.2Δ1801 and Cav1.2Δ1733 in HEK cells. However, DCT inhibited ICa,L carried by Cav1.2Δ1801 but not Cav1.2Δ1733 channels. As CaM and DCT have adjoining interaction sites on CaV1.2, we tested if exogenous CaM (CaMex) interferes with DCT current inhibition in cardiomyocytes and HEK systems. CaMex relieved DCT block in both systems and both channel variants. Addition of CaM1234 did not relieve DCT current inhibition or have an additive effect. DCT did not alter voltage dependence, or activation kinetics. The selective block of IBa,L suggested that DCT activity may be relieved under conditions of elevated Ca.

Conclusions. DCT blockade of LTCC is apparent only when Ca is not the charge carrier. Therefore, our data motivates the new hypothesis that DCT is a native reverse use-dependent inhibitor of LTCC current.

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Regulation of $Ca_V1.2$ Channels by Sphingolipids and Cholesterol. Specific Role of the Gamma Subunit

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Previoulsy (Biophys. Meetings 2010 and 2011), we showed that inactivation of L-type Ca^{2+} channels containing the γ1 subunit changes during cell cycle. The half-inactivation voltage is very negative (in the −70 - −50 mV range) in cells at the G1 phase, but also after serum starvation, or after the ER shock.

Here we report that the novel regulatory pathway is membrane-delimited because it depends on cholesterol and sphingolipids. Alterations of the membrane lipid fluidity (e.g., addition of Triton X-100) affected inactivation regardless of the γ1 subunit. However, various manipulations with membrane cholesterol, ceramide, and/or shpingosine were efficient only when the γ1 subunit was present. Although tested positively in our experimental conditions, phosphoinositides, C-8-ceramide-1-phosphate, sphingosine-1-phosphate, and glycosphingolipid GM1 did not act specifically on inactivation with the γ1 subunit.

When cholesterol was depleted from the membrane, or upon application of sphingosine, ceramide-C2, or ceramide-C8, inactivation shifted to the −70 - −50 mV range. Isomers epi-cholesterol and dihydro-ceramide-C2 did not act. Because ceramide and sphingosine strongly activate phosphatases PP1 and PP2A, phosphatase inhibitors calyculin A, or tautomycetin, were applied in order to prevent a rapid current run-down. Thus, a role of phosphorylation-dependent pathways can be excluded as well.

We compared the membrane cholesterol content in cells at different cell-cycle stages, but did not find any significant difference. However, the membrane contents of sphingosine and ceramide are well known to change during cell cycle. Therefore, we propose that the enhancement of inactivation of L-type channels by the γ1 subunit is dependent on sphingosine and ceramide. Possibly, the sphingolipids interact directly with the subunit. The large scale of changes of inactivation properties due to these interactions allows efficient spatiotemporal tuning of functional availability of L-type Ca^{2+} channels.

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Neutralizing the Charges in a Voltage Sensor Repairs Gating Perturbations in the Pore of $Ca_V1.2$

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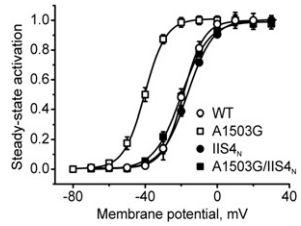
Voltage sensors initiate the closed - open transitions in the ion conducting pore. Here we consecutively neutralized the charges of the four S4 segments (IS4-IVS4) of $Ca_V1.2$. The channel construct lacking charges in IIS4 ($IIS4_N$) conducted barium currents. Surprisingly, charge neutralization accelerated channel closure and diminished shifts of the activation curves also in S6 mutants (IS6: G432W/$IIS4_N$, IIS6: A780T/$IIS4_N$, IIIS6: G1193T/$IIS4_N$, IVS6: A1503G/$IIS4_N$). Such a rescue of S6-mediated gating perturbations is exemplified for the IVS6 mutation A1503G. A1503G induced a -22.6 ± 1.2 mV shift of the activation curve to the left that was diminished by charge neutralization in IIS4. Gating disturbances induced by pore mutations upstream and downstream of G432, G1193 and A1503 were unaffected. Thermodynamic analysis reveals that segment IIS4 interacts (energetically coupled) with a ring of small homologous amino acids G432, A780, G1193 and A1503 (G/A/G/A ring, Depil et al. 2011) in all four pore forming S6 segments.



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Temperature Effects on Pharmacologic Enhancement of L-Type Calcium Current

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L-type calcium current (Cav1.2) plays a key role in modulation of multiple cardiovascular functions. Therefore, Cav1.2 calcium current assay is useful in screening discovery compounds for potential cardiovascular (CV) liabilities. L-type calcium current itself is highly sensitive to temperature. However, it is not known if drug effects on this current are influenced by temperature. Using whole-cell voltage clamp techniques, we compared the effects of room (22-24° C) and physiological (37° C) temperatures on the current in the absence and presence of a calcium current activator (TTYC) in CHO cells stably expressing L-type calcium channels. Under our experiment conditions (2 mM $[Ca^{2+}]_o$), calcium current amplitude in CHO cells was increased approximately 4-fold with warming from 24 to 37° C; the half maximal activation voltage ($V_{1/2}$) was shifted from −6.4 mV (24° C) to −17 mV (37° C). The calcium channel activator TTYC (0.3 uM, Su et al. 2010) increased peak current by 92 ± 10 %