**2193-Plat**

**Effects of Tryptophan Substitutions for Highly-Conserved Phenylalanines in the Skeletal Muscle L-Type Calcium Channel**

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The motif FxxExxxK/R is highly conserved in S2 segments of voltage-gated cation channels, including the four repeats of the $\alpha_{1s}$ subunit of the skeletal muscle L-type $Ca^{2+}$ channel, and appears to represent an important structure for voltage-sensing. Mutating the conserved S2 phenylalanine (F290) within the *Shaker* $K^+$ channel monomer to tryptophan causes a ~40 mV hyperpolarizing shift in activation (Tao et al., 2010; Lacroix & Bezanilla, 2011) due to an introduced cation-pi interaction with S4 charges (Pless et al, 2011). We made homologous F to W substitutions in repeats I (F97W), II (F475W), III (F843W) and IV (F1161W) of YFP-$\alpha_{1S}$, and tested the mutants' ability to conduct L-type current and to serve as the voltage sensor for excitation-contraction (EC) coupling after expression in *dysgenic* myotubes. Except for R1161W, each of these constructs encoded channels that were targeted to SR-membrane junctions and supported EC coupling. In contrast to the large hyperpolarizing shift in activation reported for *Shaker* F290W, no activation shift was apparent for F97W relative to YFP-$\alpha_{1S}$ ($p>0.05$) and substantial *depolarizing* shifts were evident for F475W and F843W ($V_{1/2}$ = 39.0 ± 2.9 and 44.1 ± 2.5 mV, respectively; *vs.* 32.4 ± 0.9 mV for YFP-$\alpha_{1S}$; $p < 0.001$, *ANOVA*). These depolarizing shifts in activation may have been a consequence of impeded gating charge transfer because the *Q-V* relationships for F475W and F843W were bi-sigmoidal, containing a component of charge that moved at more depolarized potentials than in YFP-$\alpha_{1S}$. Our results indicate that, despite having sequence homology, the structure and function of the voltage-sensing modules in four-repeat $Ca_V$ and $Na_V$ (Pless et al., this meeting) channels diverge significantly from those of homotetrameric $K_V$ and bacterial $Na^+$ channels. Supported by NIH AR055104 (K.G.B) and AG038778 (R.A.B.), and MDA4319 (K.G.B.)

**2194-Plat**

**The Distal Carboxy Tail (DCT) of $Ca_V1.4$ Modulates More than $Ca^{2+}$/CaM-Dependent Inactivation (CDI)**

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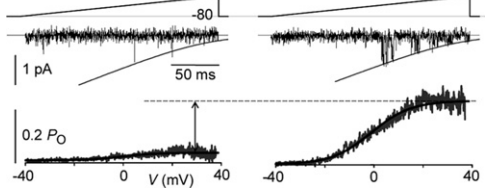
$Ca_V1.4$ L-type $Ca^{2+}$ channels populate retinal ribbon synapses, and mediate tonic neurotransmitter release. Mutations in the CACNA1F gene encoding $Ca_V1.4$ are implicated in congenital stationary night blindness (CSNB2). The K1591X CSNB2 mutation yields a premature stop that deletes the DCT of $Ca_V1.4$. Interestingly, the DCT contains a module that competes with $Ca^{2+}$-free calmodulin (apoCaM) for binding at the IQ-domain of $Ca_V1.4$, thereby tuning channel affinity for apoCaM (*Nature* **463**:968). Since only channels 'charged' with apoCaM exhibit $Ca^{2+}$/CaM-mediated inactivation (CDI), wildtype $Ca_V1.4$ channels exhibit little or no CDI, while deleting the DCT yields a resurgence of CDI. The latter effect in K1591X channels has suggested that pathogenesis involves abnormally diminished $Ca^{2+}$ influx with resulting impairment of photoreceptor signaling. Here, however, we observe a dramatic and unrecognized effect of DCT deletion. Single-channel recordings indicate that wild-type $Ca_V1.4$ feature diminutive open probability $P_o$ (**A.** Top, exemplar single-channel trace during voltage ramp. Bottom, $P_o$-*V* relation averaged over multiple patches). By contrast, K1591X channels exhibit strikingly enhanced $P_o$ (**B**). Hence, this form of CSNB2 likely involves an unexpected $Ca^{2+}$ overload phenomenon, raising the possibility of therapeutics involving $Ca_V1.4$ channel blockade.




**2195-Plat**

**Analysis of Competition between CaBP1 and Calmodulin on the Voltage-Gated Calcium Channel Cav1.2**

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Voltage-gated calcium channel ($Ca_V$) activity is regulated by calcium sensors including calmodulin (CaM) and calcium-binding protein 1 (CaBP1). CaBP1 inhibits CaM-mediated calcium-dependent inactivation (CDI). We investigated the origins of functionally important differences between CaM and CaBP1 by creating a number of CaM/CaBP1 chimeras. The exchange of C-terminal lobes between CaM and CaBP1 is accompanied by few functional changes, suggesting CaBP1 and CaM C-lobe can substitute for each other. In contrast, we identified the linker and the N-terminal lobe of CaBP1 as elements that set it functionally apart from CaM. CaBP1 and CaM are thought to modulate $Ca_V$ function by competing for binding to the $Ca_V$ C-terminal IQ-domain, but this assumption has never been tested directly. By determining $Ca_V1.2$ CDI under conditions with different ratios of CaM and CaBP1, we demonstrate direct competition between CaBP1 and CaM for its $Ca_V1.2$ binding site. In order to extend our analysis of CaBP1/CaM competition we used isothermal titration calorimetry to determine the affinity of both CaM and CaBP1 in both calcium-bound and apo-states for the IQ domain, suggesting that competition occurs mainly in the apo-state. Analysis of an IQ domain mutant shows unexpected differences between CaM and CaBP1 in IQ domain binding, suggesting a possible mechanism for their different functional effects on $Ca_V1.2$ inactivation. Overall, the data reveal those parts of CaBP1 that set it functionally apart from CaM and provide a framework for understanding how CaBP1 and CaM differentially regulate CDI on $Ca_V1.2$.

**2196-Plat**

**Nanometer Resolution of $Ca_v$-Generated $Ca^{2+}$ Gradients**

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Diffusion theory predicts that each pA of $Ca^{2+}$ flux through a voltage-gated $Ca^{2+}$ channel ($Ca_V$) should generate ~100 μM free [$Ca^{2+}$] in the channel nanodomain, with steep spatial gradient and little sensitivity to intracellular $Ca^{2+}$ buffers. This is widely quoted, and bears on synaptic transmission, activity-dependent plasticity, and excitation-contraction coupling. Yet, experimental verification has been difficult because diffusible $Ca^{2+}$ indicators report space-averaged [$Ca^{2+}$], and $Ca_V$-tethered fluorescent indicators cannot distinguish between active and silent channels. Here, we used $Ca^{2+}$/calmodulin-dependent inactivation (CDI) of $Ca_V1.3$ channels themselves as a nearfield indicator of nanodomain [$Ca^{2+}$], noting that CDI provides an ionic readout based only on active channels. We calibrated the [$Ca^{2+}$]-sensitivity of CDI by monitoring the decay of $Li^+$ currents following spatially-uniform $Ca^{2+}$ uncaging steps. We then ran $Ca^{2+}$ through the same channels to generate local [$Ca^{2+}$] gradients, and titrated the external $Ca^{2+}$ concentration until CDI diminished ~50%. The corresponding unitary flux was resolved through a combination of single-channel and whole-cell recordings, enabling determination of the relationship between unitary $Ca^{2+}$ flux and nanodomain [$Ca^{2+}$]. Contrary to theory, nanodomain [$Ca^{2+}$] signals are larger and more buffer-sensitive than expected: ~800 μM/pA in 10 mM intracellular BAPTA, and ~200 μM/pA in 60 mM BAPTA. These findings indicate that diffusion of free $Ca^{2+}$ ions is ~10-fold slower in the nanodomain versus other regions of the cell, and that calmodulin (the $Ca^{2+}$-sensing subunit for CDI) is ~7 nm from the channel pore. Our results suggest that the nanodomain is a tortuous environment, where a $Ca^{2+}$ ion would collide with an obstacle in 9 out of every 10 of its Brownian movements. Under physiological buffering, we predict that $Ca_V$s, by virtue of their crowded nanodomains, convert small $Ca^{2+}$ fluxes into enormous $Ca^{2+}$ concentrations, ~1 mM/pA over distances of 10-20 nm.

**2197-Plat**

**Inhibitory Cav2.2 Peptides Effects in Superior Cervical Ganglion Neurones**

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We have been developing inhibitory CaV2.2 peptides based on reported interaction sites between voltage-dependent Ca2+ channels (VDCCs) and G protein βγ subunits. We have shown that an alpha interaction domain CaV2.2[377-393] 'AID peptide' and a CaV2.2[45-55] amino terminal 'NT peptide' act to reduce synaptic transmission via a blockade of VDCCs in superior cervical ganglion neurons (SCGNs) (Bucci et al., 2011, J Physiol 589:3085-101). Here, we compare the effects of the AID peptide and an 'AID W-A peptide' containing a (W391A) mutation to a tryptophan residue previously implicated in VDCC function (both peptides used at 1 mM).

In SCGN synapses, EPSP amplitude was significantly reduced by presynaptic injection of AID (20.9 ± 3.9 %, n=6, P<0.05) and AID W-A (34 ± 3.9 %, n=6, P<0.05) peptides; with increased inhibitory effects for the AID W-A peptide (P<0.05 vs AID). In isolated SCGNs, Ca2+ current-density at 0 mV (control: 40 ± 6.5 pA/pF, n=9) was significantly reduced by AID (25 ± 4.6 pA/pF, n=9, P<0.05) and AID W-A (14 ± 2.7 pA/pF, n=9, P<0.001) peptides; representing increased AID W-A peptide inhibitory effects (P<0.05 vs AID). Consistent

with these effects, intracellular Ca2+ accumulation in response to depolarizing voltage steps to 0 mV (control: 60.8 ± 3.0 nM, n=7) was significantly reduced by AID (29.3 ± 1.6 nM, n=7, P<0.001) and AID W-A (18.6 ± 3.4 nM, n=7, P<0.001) peptides; again representing increased inhibitory AID W-A peptide effects (P<0.05 vs AID). Under these conditions, both AID and AID W-A peptides also attenuated G-protein modulation of Ca2+ current (as induced by 1 μM somatostatin).
These data are consistent with inhibitory CaV2.2 peptides acting to reduce synaptic transmission due to a direct effect on presynaptic VDCCs. The increased inhibitory effect of the AID W-A peptide may represent a starting point to develop inhibitory agents in aberrant CaV2.2 signalling pathways, such as in nociception.

**2198-Plat**
**Ca2+ Signaling Amplification by Oligomerization of L-Type Cav1.2 Channels**
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Ca2+ influx via L-type CaV1.2 channels is essential for multiple physiological processes including gene expression, excitability, and contraction. Amplification of the Ca2+ signals produced by the opening of these channels is a hallmark of many intracellular signaling cascades, including excitation-contraction (EC) coupling in heart. Using optogenetic approaches, we discovered that CaV1.2 channels form clusters of varied sizes in ventricular myocytes and that physical interaction between these channels via their C-tails renders them capable of coordinating their gating thereby amplifying Ca2+ influx. Light-induced fusion of wild type (WT) CaV1.2 channels with channels carrying a gain-of-function mutation that causes arrhythmias and autism in humans with Timothy syndrome (CaV1.2-TS) increased Ca2+ currents, diastolic and systolic Ca2+ levels, contractility, and the frequency of arrhythmogenic Ca2+ fluctuations in ventricular myocytes. Our data indicate that these changes Ca2+ signaling resulted from CaV1.2-TS increasing the activity of adjoining WT CaV1.2 channels via protein-to-protein interactions. Our data support the novel concept that oligomerization of CaV1.2 channels can control the amplification of Ca2+ influx in excitable cells.

# Platform: Interfacial Protein-Lipid Interactions II

**2199-Plat**
**Synaptotagmin C2 Domain Membrane Targeting: Kinetic and Mechanistic Diversity Among Isoforms from Different Cell Types**
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Synaptotagmin (Syt) triggers $Ca^{2+}$-dependent membrane fusion during secretion via its tandem C2 domains, termed C2A and C2B. The seventeen known human isoforms are active in different secretory cell types, including neurons (SytI and others) and pancreatic β cells (SytVII and others). Here, quantitative fluorescence measurements reveal notable differences in the membrane docking affinities, kinetics, and molecular driving forces for C2A and C2B domains from SytI and SytVII, using vesicles comprised of physiological target lipid mixtures. In agreement with previous studies, the $Ca^{2+}$ sensitivity of membrane binding is greater for both domains from SytVII than for their counterparts in SytI. We demonstrate that for C2A, this increased sensitivity is due to a stronger SytVIIC2A membrane interaction, which involves substantial contribution from the hydrophobic effect. Association and dissociation rate constants for both SytVII domains are found to be significantly slower than their counterparts in SytI. For SytVIIC2A, the dissociation rate constant is ~50-fold slower than SytIC2A and is reminiscent of the $cPLA_2C2$ domain that is known to insert deeply into membranes. Addition of sodium sulfate decreases the dissociation rate of SytVIIC2A but not SytIC2A, further indicating that hydrophobic contacts play a major role in SytVIIC2A membrane docking. Thus, SytVIIC2A docks to membranes via both hydrophobic and electrostatic interactions, while the membrane docking interaction of SytIC2A is predominantly electrostatic. The inclusion of phosphatidylinositol-4,5-bisphosphate ($PIP_2$) in membrane mixtures leads to increased affinity and slower dissociation for both C2B domains, but has minimal effects on C2A domains. Overall, highly homologous domains from these two proteins exhibit distinct mechanisms of membrane binding that may reflect their functions in different cell types.

**2200-Plat**
**How PIP2 Lipids Regulate the Position and Phosphorylation of the Syntaxin N-Terminus**
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Syntaxin, a member of the family of soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins can bind and regulate plasma membrane ion channels and neurotransmitter transporters (NSS). Studies of such mechanisms for the dopamine transporter (DAT) have established the N-terminal segment of Syntaxin as the site of direct interactions, and have shown the critical role of highly charged PIP2 lipids in regulating Syntaxin-DAT interactions. We used a computational approach that combines mesoscale continuum modeling of protein-membrane interactions with all-atom molecular dynamics (MD) simulations to compare conformational states of Syntaxin in complex with PIP2-enriched and PIP2-depleted membranes. Our mesoscale approach is based on non-linear Poisson-Boltzmann theory of electrostatics and diffusion-like Cahn-Hilliard dynamics that makes possible the quantitative tracking of lipid-type demixing in the membrane due to the interaction with the protein. The calculations with this method identified strong electrostatic interactions of specific sites of Syntaxin with PIP2 lipids that diffused to their vicinity. MD simulations of the resulting system established that as many as five PIP2 lipid molecules can simultaneously bind Syntaxin. The attending segregation of PIP2 lipids appears to have a dramatic effect on the positioning of the Syntaxin N-terminal segment with respect to the membrane/water interface. These results are discussed in the context of the suggested role of PIP2 lipids in regulating Syntaxin-DAT interactions by modulating phosphorylation of Syntaxin at its N-terminus.

**2201-Plat**
**Structure and Kinetics of PTEN Tumor Suppressor Association with Lipid Membranes**
**Siddharth Shenoy**[1], Prabhanshu Shekhar[1], Frank Heinrich[1,2], Hirsh Nanda[1,2], Joseph Curtis[2], Katrice King[3], Arne Gericke[3], Alonzo Ross[4], Mathias Lösche[1,2].
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PTEN is the second most commonly mutated protein in human cancer [1]. The PTEN-PI3K phosphorylation switch regulates cell growth and survival by controlling the levels of $PI(3,4,5)P_3$ in the plasma membrane. PTEN's association with the membrane is critical for bringing its active site in close proximity to the substrate, $PI(3,4,5)P_3$. The crystal structure of a truncated PTEN was determined [2], however, the structure of membrane-bound PTEN remains unknown. In this work, we perform systematic binding studies of PTEN with membranes and report first steps toward a structural characterization of PTEN associated with bilayers.
We compared the binding affinities of wt PTEN, the truncated PTEN [2] and two point mutants, C124S and H93R, to lipid membranes with various anionic lipid (PS, $PI(4,5)P_2$ and $PI(3,4,5)P_3$) compositions using Surface Plasmon Resonance (SPR). PS and $PI(4,5)P_2$ show strong cooperativity in binding to wt PTEN while $PI(4,5)P_2$ and $PI(3,4,5)P_3$ show independent binding to the catalytically inactive C124S mutant. The H93R mutation is spatially separated from the active site as well as the membrane binding motifs, yet results in altered affinities to PS and $PI(4,5)P_2$. The truncated PTEN mutant has an increased affinity to PS-containing membranes over wt PTEN due to an increase in its net positive charge. Neutron reflectivity (NR) experiments were performed to characterize the structure of the PTEN-membrane complex. We observe minimal penetration of the proteins into the lipid headgroup region, indicating that protein association occurs only with the membrane surface. Small, yet significant differences in the NR profiles emphasize the role the point mutations have in altering PTEN's association with the membrane. Molecular dynamics and coarse-grained simulations are currently being performed to interpret structural and orientational details in the NR data.
[1] Nature (2006) 441, 424-430.
[2] Cell (1999) 99, 323-334.

**2202-Plat**
**Exploring Fluorescence Lifetime and Homo-FRET Measurements to Monitor Lysozyme Oligomerization in Anionic Lipid Membranes: Relation to "Amyloid-Like" Fibril Formation**
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