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Authors
Man, Kwun Nok Mimi
Bartels, Peter
Horne, Mary C
et al.

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Tissue-specific adrenergic regulation of the L-type Ca^{2+} channel $Ca_V1.2$

Kwun Nok Mimi Man[#], **Peter Bartels**[#], **Mary C. Horne**[*], **Johannes W. Hell**[*]

Department of Pharmacology, University of California, Davis, CA 95616

Abstract

Ca^{2+} influx through the L-type Ca^{2+} channel Cav1.2 triggers each heartbeat. The fight or flight response induces the release of the stress-response hormone norepinephrine to stimulate β adrenergic receptors, cAMP production, and protein kinase A activity to augment Ca^{2+} influx through Cav1.2 and consequently cardiomyocyte contractility. Emerging evidence shows that Cav1.2 is regulated by different mechanisms in cardiomyocytes compared to neurons and vascular smooth muscle cells.

The L-type Ca^{2+} channel Cav1.2, which is the most prevalent Ca^{2+} channel in the heart, mediates the initial Ca^{2+} influx into cardiomyocytes that subsequently induces Ca^{2+} release from internal stores (1). Cytoplasmic Ca^{2+} concentrations eventually reach a threshold for triggering cardiomyocyte contraction. The closely-related L-type channel Cav1.3 plays an important role in initiating the heart beat in the sinoatrial node, which serves as the primary pacemaker for the heart (2). Cav1.2 also mediates Ca^{2+} influx into neurons, which governs gene expression (3, 4) and excitability (5, 6).

The stress hormone norepinephrine increases the rate and strength of heart muscle contraction during the fight or flight response. The increase in strength is largely due to upregulation of the activity of the cardiac Cav1.2 by norepinephrine (7). Because of the central physiological role and hence the long-lasting interest in and focus on the regulation of Cav1.2, it is considered one of the holy grails of channel regulation. The timely work of Marx and colleagues delineated one major regulatory mechanism in heart muscle (8). They found that stimulation of β adrenergic receptors (β AR) by norepinephrine leads to PKA-mediated phosphorylation of the small G-protein Rad. This modification induces the release of Rad from Cav1.2. Because Rad is a potent negative regulator of the cardiac Cav1.2, its displacement from the complex results in disinhibition of channel activity (8).

The first milestone in deciphering this mechanism was the identification of Ser^{1928} in the C-terminus of Cav1.2 of the pore-forming α subunit of Cav1.2 as the main PKA phosphorylation site in the channel (9) (Figure 1). PKA is activated by cAMP, a second

[*]Co-senior authors: mhorne@ucdavis.edu ; jwhell@ucdavis.edu.

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[#]Co-first authors

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messenger that is produced upon the activation of the stimulatory trimeric Gs protein and adenylyl cyclase following stimulation of β ARs (Figure 1). PKA is the primary downstream kinase mediating norepinephrine signaling through β ARs. However, definitive evidence for a critical role of this signaling paradigm had been difficult to establish because ectopic expression of Cav1.2 in heterologous cell lines does not reliably reconstitute this regulatory pathway (10). Early evidence from studies in HEK293 cells supporting a role for Ser^{1928} in increasing Cav1.2 activity was limited or required complex expression system manipulations (4, 11, 12). Still, several studies demonstrating a robust increase in Ser^{1928} phosphorylation in heart (13) and other tissues and especially brain, where Cav1.2 plays important roles in regulating neuronal functions (14, 15) buttressed the notion that Ser^{1928} phosphorylation is a critical event in governing Cav1.2 activity.

In 2008, Hofmann and colleagues reported that a S1928A knock-in mutation of the Cav1.2 α-subunit did not affect the peripheral fight or flight response, arguing against the importance of this PKA target site in mediating β adrenergic signaling, at least in heart muscle (16). Coincidentally, Catterall and colleagues identified Ser^{1700} as a second PKA site in the Cav1.2 C-terminus (17) (Figure 1). By titrating the amount of the A kinase anchoring protein AKAP15/18, which recruits PKA to Cav1.2, they defined the conditions under which an increase in Cav1.2 activity and the consequent coupling of gating charge movement to pore opening could be reliably observed in HEK293 cells (17). Too little AKAP15/18 does not provide sufficient attachment sites to enable PKA association with Cav1.2 and too much may act as a sink to sequester PKA and reduce its association in Cav1.2. By titrating AKAP15/18 amounts, Fuller *et al.* reproducibly obtained PKA-mediated increases in Cav1.2 activity in HEK293 cells. Subsequent work in S1700A knock-in mice indicated that this phosphorylation site contributes to increasing Cav1.2 activity in heart tissue, but did not account for the full norepinephrine effect (18). Further clouding the picture were observations from expression of different Cav1.2 mutants in vivo that suggested that Ser^{1700} did not play a substantial role of in norepinephrine regulation of cardiac Cav1.2 activity (19). Moreover, Liu *et al* showed that mutating all intracellular consensus sites for PKA in Cav1.2 did not abrogate the increase in activity by β AR stimulation in the heart (8). The main factor for the regulation of the cardiac Cav1.2 had not been identified.

The next turning point was the recognition that Ser^{1928} is part of the attachment site for the β_2 AR on Cav1.2 (20). This signaling complex also contains Gs, adenylyl cyclase, AKAPs, and PKA for highly localized regulation of Cav1.2 activity in neurons (21, 22) and cardiomyocytes (23) (Figure 1). Repetitive stimulation of the β_2 AR leads to a temporary (~5 min) displacement of the β_2 AR from Cav1.2 (20), an effect that was absent in S1928A knock-in mice. This mechanism creates a refractory period during which this signaling pathway cannot be re-stimulated (20), suggesting a potential negative feedback loop. However, further analysis of S1928A knock-in mice revealed that in contrast to cardiomyocytes, Ser^{1928} is required for upregulation of Cav1.2 by β_2 AR stimulation in hippocampal neurons (24). Parallel experiments confirmed that β-adrenergic stimulation of Cav1.2 is not affected in cardiomyocytes from S1928A knock-in mice (24). At the same time upregulation of Cav1.2 activity in VSMCs upon stimulation of the Gs/PKA-coupled adenosinic P_2Y_{11} receptor in vascular smooth muscle cells (VSMCs) also depended on Ser^{1928} (25, 26).

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The work by Marx and his colleagues is based on a proximity assay in which Cav1.2 was tagged with ascorbate peroxidase, which biotinylates proteins within a 20 nm region around the channel (8). Biotinylation of Rad was decreased upon β AR stimulation, which was the strongest effect for any of the biotinylated proteins and which suggested that β AR stimulation robustly displaces Rad from the Cav1.2 complex. Rad belongs to the RGK family of small G proteins, which also includes Rem, Rem2 and Gem/Kir. These G proteins inhibit Cav1.2 activity (27-29). The authors found that co-expression of Rad reduced Cav1.2 activity in HEK293 cells. Stimulation of adenylyl cyclase augmented Cav1.2 activity in the presence of Rad, but had no further effect on the already fairly high activity of Cav1.2 in the absence of Rad. Mutating the putative PKA sites Ser^{272} and Ser^{300} at the extreme C-terminus of Rad, a region that binds to negatively charged phospholipids such as PIP_2, abrogated the regulation by cAMP. This was consistent with the importance of the interaction of the C-terminus of Rem with the membrane for Cav1.2 inhibition (30, 31). Liu *et al.* showed that PKA-mediated phosphorylation displaced Rad from the auxiliary β subunit in the Cav1.2 complex, thereby disinhibiting the channel activity (8). This was coherent with an essential role of β subunits in upregulation of Cav1.2 activity by β adrenergic signaling in the heart (32).

The mechanism of Cav1.2 inhibition by Rad is most likely as complex as that shown for the closely related Rem. Rem impairs the functional availability of Cav1.2 in cardiomyocytes in three ways (Figure 2) (31). Firstly, Rem reduces surface abundance, possibly by binding to auxiliary β subunits in Cav1.2, which otherwise augment trafficking of Cav1.2 through the secretory pathway (31). Secondly, Rem reduces the open probability (Po) of Cav1.2 channels. Thirdly, Rem impairs the movement of the voltage sensors in Cav1.2. The effects of Rem and Rad on surface localization and Po depend on their binding to the β subunit whereas those on gating charge movement do not (33). Rem can directly bind to the N- and C-terminus of the central pore forming $\alpha_1$1.2 subunit of Cav1.2 (30, 33) (Figure 1). How Rad impairs Cav1.2 functions is currently unclear. However, it is tempting to speculate that it could affect α-actinin binding to the IQ motif in the membrane-proximal portion of the C-terminus (PCT) of $\alpha_1$1.2 because this interaction increases the same three parameters, namely surface abundance and localization (34, 35), gating charge movement, and its coupling to pore opening (with the latter two determining Po) (36). In fact, Cav1.2 has low activity in the absence of α-actinin binding – the Po of α-actinin binding-deficient Cav1.2 mutants is ~15-20% of wild type Cav1.2 - but becomes primed for activation when at its designated location at the cell surface by α-actinin, an effect that minimizes unintended Ca^{2+} flux activity at inappropriate locations such as the secretory pathway (36). Binding of RGK proteins instead of α-actinin to Cav1.2 could contribute to keeping the channel inactive in the secretory pathway. In turn, α-actinin binding to the PCT could impair binding of Rad to the α_1 or β subunit and thereby augment Po. An antagonistic effect of RGK proteins on α-actinin binding would also explain the opposite effects of these two proteins on the surface abundance and Po of Cav1.2 (31, 36).

Why is phosphorylation of Ser^{1928} critical in neurons but not cardiomyocytes? One key difference is that upregulation of Cav1.2 activity by β adrenergic signaling is exclusively mediated by the β_2 AR in neurons (24) and mostly by the β_1 AR in cardiomyocytes (37, 38). However, the β_2 AR can contribute to upregulation of Cav1.2 activity under certain

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conditions in the heart (23, 39), especially under pathological conditions such as heart failure (37, 38). But why would the β_1 AR and the β_2 AR differentially couple to Cav1.2 regulation in heart and brain? The answer might lie in differences in the microenvironment, which could affect Cav1.2 conformation and thereby its regulation. In heart but not in brain, most of Cav1.2 appears to be located within membrane rafts or caveolae (22, 23). In fact, pharmacological disruption of rafts as well as caveolin-3 knockdown impairs the limited regulation of Cav1.2 by the β_2 AR in heart that occurs upon pertussis toxin blockade of the inhibitory trimeric Gi protein (23, 39). The requirement of the Gi block to detect this β_2 AR regulation is likely due to the ability of the β_2 AR to switch from Gs coupling to Gi coupling (40). That such a Gi block is not necessary in hippocampal neurons, where signaling is exclusively mediated by the β_2 AR and not the β_1 AR and apparently outside of membrane rafts, fits with the notion that the microenvironment could affect Cav1.2 conformation and regulation. An additional reason could be the association of Cav1.2 with different sets of auxiliary subunits or their isoforms. For instance, there are four different genes encoding β subunits, each exhibiting multiple splice isoforms (10), which could impart different properties and conformations to the Cav1.2 complexes. In fact, emerging evidence indicates that alterations in the binding site for β subunit between domains I and II affect regulation of Cav1.2 activity by PKA (41).

How could the microenvironment influence Cav1.2 regulation? The ~660 residue long C-terminus of $\alpha_1$1.2 is functionally divided into roughly two ~ 300 residue long portions, the PCT and the membrane-distal one (DCT). These two domains interact through salt bridges between Arg^{1696}/Arg^{1697} and Glu^{2103}/Glu^{2106}/Asp^{2110} (42) (Figure 1). Deletion of DCT results in increased Cav1.2 activity (42, 43), an effect that can be reversed by expression of the DCT as a separate polypeptide (42). Arg^{1696} and Arg^{1697} form a consensus sequence for the phosphorylation of the nearby Ser^{1700} by PKA, which augments Cav1.2 activity (17). Collectively, these findings suggest that Ser^{1700} phosphorylation disrupts the PCT-DCT interaction (42) and thereby augments Cav1.2 activity. Similarly, Ser^{1928} phosphorylation could increase Cav1.2 activity (20) by disrupting the PCT-DCT interaction (Figure 1). The degree to which the DCT impairs the channel activity could depend on the microenvironment – perhaps the interaction between the PCT and DCT is prominent outside (neurons) but not inside membrane rafts (cardiomyocytes). Such a cell type dependence on membrane rafts would predict that phosphorylation of Ser^{1700} or Ser^{1928} results in disinhibition of Cav1.2 in neurons where this interaction curbs the channel activity but not in cardiomyocytes where this interaction might be less prevalent (Figure 1). Ser^{1700} phosphorylation appears to be less important than Ser^{1928} phosphorylation in upregulating Cav1.2 activity in neurons. A form of long-lasting synaptic potentiation that is induced by coincident prolonged synaptic activation at the 5-8 Hz theta rhythm and β adrenergic stimulation and involves Cav1.2 requires phosphorylation of Ser^{1928} but not that of Ser^{1700} (20, 24).

The dependence of β adrenergic upregulation of Cav1.2 in neurons but not in cardiomyocytes on Ser^{1928} phosphorylation could theoretically be due to differential regulation of this phosphorylation event by β_2 AR compared to β_1 AR signaling, leading to variant target phosphorylation in neurons compared to cardiomyocytes. This possibility would be consistent with the β_2 AR but not the β_1 AR being part of the Cav1.2/Gs/adenylyl

cyclase/PKA signaling complex (22, 23). However, β AR stimulation leads to a robust increase in Ser^{1928} phosphorylation not only in neurons (20) but also in cardiomyocytes (13), arguing that this possibility is not the main explanation for the tissue-specific differential functions of Ser^{1928} .

Another interesting twist is the observation that the β_2 AR locally restricts signaling by the β_1 AR in cardiomyocytes to Cav1.2 (44). This restriction prevents β_1 AR-induced phosphorylation of the juxtaposed ryanodine receptor, which mediates Ca^{2+}-induced Ca^{2+} release (CICR) after Ca^{2+} influx through Cav1.2. Upregulation of CICR from the sarcoplasmic reticulum but not of Cav1.2 currents upon stimulation of the β_1 AR with the non-selective β AR agonist isoproterenol is impaired only by strong activation of the β_2 AR by co-application of the β_2 AR selective agonist salbutamol but not by the modest activation induced by isoproterenol alone (44). This restriction of β_1 AR to the nanospace around Cav1.2 by the β_2 AR appears to depend on the PDE4 family of phosphodiesterases (PDEs) and the C-terminus of the β_1 AR, because this effect is prevented by the PDE4 selective inhibitor rolipram or injection of a peptide corresponding to the C-terminal 36 residues of the β_1 AR, which includes its binding site for PDZ-domain containing scaffolding proteins. This restraint of β_1 AR signaling is also abrogated by injection of β-arrestin-1 (but not β-arrestin-2) and an inhibitor of the G protein coupled receptor kinase GRK2. Further experiments showed that activation of the β_2 AR but not β_1 AR caused GRK2 translocation to Caveolin-3 containing membrane sites, consistent with the recruitment to membrane rafts where Cav1.2 resides in cardiomyocytes. In knock-in mice expressing a form of the β_1 AR in which the three serine residues near the extreme C-terminus that are phosphorylated by GRKs are replaced with alanine residues, which would be expected to abolish arrestin binding, the spatial restriction of β_1 AR signaling by β_2 AR signaling is lost (44). Collectively, these results suggest that β_2 AR activation results in β_1 AR phosphorylation by GRKs, and that binding of β-arrestin-1 to the β_1 AR compartmentalizes cAMP signaling by the β_1 AR to the immediate Cav1.2 environment. In the presence of β_2 AR signaling, β_1 AR signaling downstream of cAMP/PKA does not upregulate CICR by the juxtaposed ryanodine receptor, which is only a few nanometers away and otherwise effectively regulated by β_1 AR signaling (1). β_2 ARs can exist as monomers and dimers, which become differentially phosphorylated upon isoproterenol stimulation at Ser^{365}/Ser^{366} only in monomers by GRKs or at Ser^{261}/Ser^{262} only in dimers by PKA. The monomers but not dimers undergo agonist-induced endocytosis (45). Given that Ser^{261}/Ser^{262} phosphorylation is required for upregulation of Cav1.2 by β_2 AR signaling (45) it appears likely that the dimeric form of the β_2 AR associates with and regulates Cav1.2. These observations support the notion that the β_2 AR adopts different conformations without ligand stimulation, thus allowing its selective interaction with downstream targets and thereby potentially creating defined microenvironments.

Could Rad or another RGK also regulate Cav1.2 in neurons? Rad, Rem, and Gem are all poorly expressed in brain (46). However, there is a subset of neurons in which Gem2 is abundant (47). Thus, it seems likely that in most neurons, RGK proteins are simply not present in sufficient amounts to interact with and inhibit Cav1.2 activity. In neurons in which Gem2 is abundant, β_2 AR signaling-mediated upregulation of Cav1.2 may require phosphorylation of both Ser^{1928} as well as Rem2.

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Rad and Rem can also suppress the activity of the L-type Ca^{2+} channel Cav1.3 and the N-type Ca^{2+} channel Cav2.2 and cAMP signaling can disinhibit their channel activities (8). The regulation of Cav1.3 by Rad is relevant for cardiac function, because Cav1.3 is a major contributor to the pacemaker activity of the sinoatrial node and thereby our heart rate (2). Accordingly, Rad-dependent regulation of Cav1.3 likely contributes to the increase in heart rate during the fight or flight response.

By defining the key phosphorylation sites in the Cav1.2 complexes for norepinephrine signaling, the journey to understand norepinephrine-mediated regulation of Cav1.2 activity has reached a prominent milestone. Yet, we have only arrived at the next course of questions now awaiting answers. Perhaps the most crucial questions at this point are: How does Ser^{1928} phosphorylation translate into increased Cav1.2 activity, and why does this phosphorylation lead to increased channel activity in neurons but not cardiomyocytes? The next level of mechanistic insight into the fascinating and remarkable complexity of Cav1.2 regulation is now ripe for further exploration.

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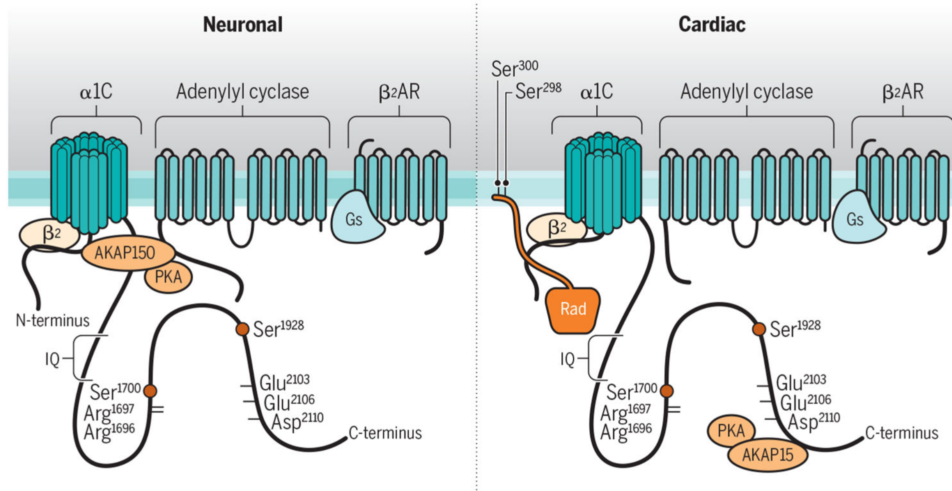

Figure 1: Differential regulation of Cav1.2 by β AR stimulation in neurons and cardiomyocytes.
Models depict the hypothesized overall structural arrangements of part of the β_2 AR/Cav1.2 signaling complex as might be prevalent in neurons (left) and cardiomyocytes (right). Cav1.2 is thought to mostly reside outside lipid rafts in neurons (darker membrane) and in lipid rafts in cardiomyocytes (lighter membrane) (22, 23). Rad is present predominantly in cardiomyocytes and might bind to the β subunit and the N-terminus and PCT of the α_1 subunit of Cav1.2, either through multiple interactions as depicted or in multiple copies (not depicted). AKAP150 and AKAP15 seem to be the predominant AKAP in neurons and cardiomyocytes, respectively. AKAP150 links both adenylyl cyclase and PKA to Cav1.2 and has multiple interaction sites with Cav1.2 (15). AKAP15 is shorter than AKAP150 and may link only PKA to Cav1.2. How AC is linked to Cav1.2 when AKAP15 is in the Cav1.2 complex is unclear. The PCT and DCT are depicted in a ‘closed’ conformation in the left model due to salt bridges between Arg^{1696} and Arg^{1697} in the PCT and Glu^{2103}, Glu^{2106} and Asp^{2110} in the DCT and in an ‘open’ conformation in the right model, perhaps due to differences in the environment of Cav1.2 (inside compared to outside of rafts) or associated proteins. Arg^{1696} and Arg^{1697} are part of the PKA consensus site for phosphorylation of Ser^{1700}, which disrupts these salt bridges. Ser^{1928} is the main PKA phosphorylation site in $\alpha_1$1.2. For simplicity, the linkers between the transmembrane segments of the α_1 subunit of Cav1.2 are not shown.

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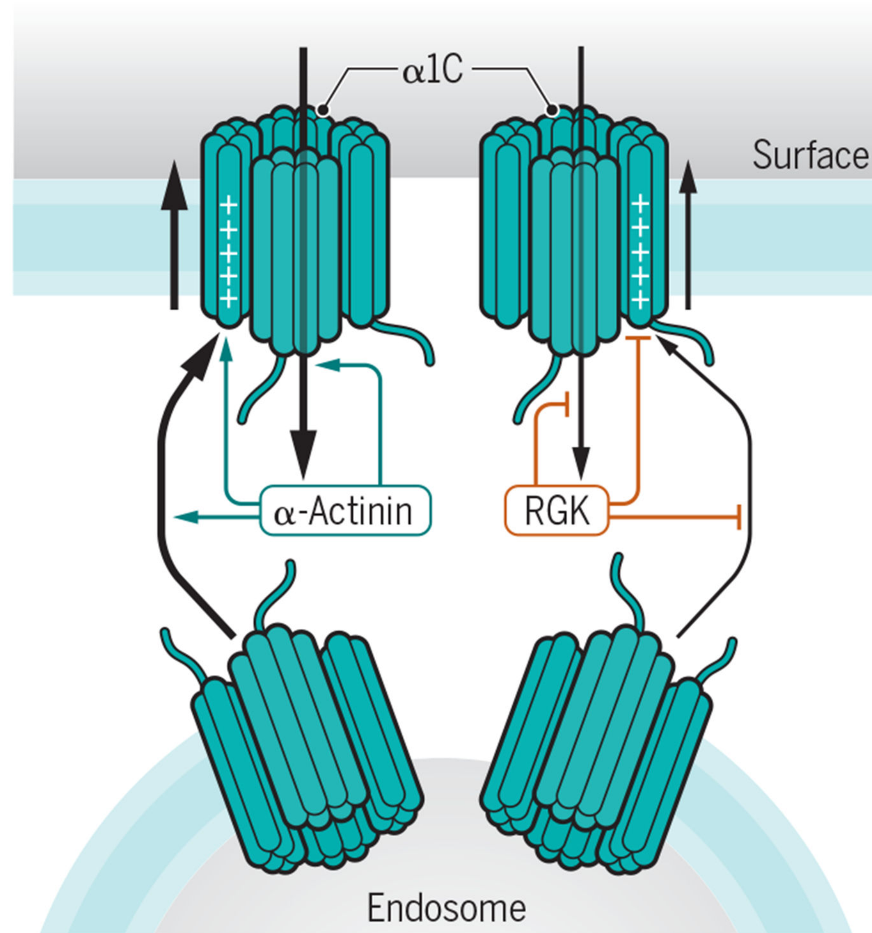

Figure 2: Antagonistic effects of Rem and α-actinin on Cav1.2.

Schematic depiction of α-actinin promoting (green arrows) and Rem antagonizing (red lines) three major parameters that affect Cav1.2 functions: surface abundance, movement of the voltage sensing S4 transmembrane helices, which carry the gating charges, and coupling of gating movements to pore opening. Impaired coupling has been directly determined for loss of α-actinin binding to Cav1.2 (36) and is suggested by analogy for Rem binding (31, 33). For simplicity, the linkers between the transmembrane segments of the α_1 subunit of Cav1.2 are not shown.