Molecular Determinants of the Differential Modulation of Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 by Nifedipine and FPL 64176\textsuperscript{S}

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ABSTRACT

Nifedipine and FPL 64176 (FPL), which block and potentiate L-type voltage-gated Ca\textsuperscript{2+} channels, respectively, modulate Ca\textsubscript{v}1.2 more potently than Ca\textsubscript{v}1.3. To identify potential strategies for developing subtype-selective inhibitors, we investigated the role of divergent amino acid residues in transmembrane domains IIIS5 and the extracellular IIIS5-3P loop region in modulation of these channels by nifedipine and FPL. Insertion of the extracellular IIIS5-3P loop from Ca\textsubscript{v}1.2 into Ca\textsubscript{v}1.3 (Ca\textsubscript{v}1.3+) reduced the IC\textsubscript{50} of nifedipine from 289 to 101 nM, and substitution of S1100 with an A residue, as in Ca\textsubscript{v}1.2, accounted for this difference. Substituting M1030 in IIIS5 to V in Ca\textsubscript{v}1.3+ (Ca\textsubscript{v}1.3+V) further reduced the IC\textsubscript{50} of nifedipine to 42 nM. FPL increased current amplitude with an EC\textsubscript{50} of 854 nM in Ca\textsubscript{v}1.3, 103 nM in Ca\textsubscript{v}1.2, and 99 nM in Ca\textsubscript{v}1.3+V. In contrast to nifedipine block, substitution of M1030 to V in Ca\textsubscript{v}1.3 had no effect on potency of FPL potentiation of current amplitude, but slowed activation in the presence and absence of 10 μM FPL. FPL had no effect on deactivation of Ca\textsubscript{v}1.3/dihydropyridine-insensitive (DHPi) channel, with very low sensitivity to nifedipine block (IC\textsubscript{50} ~93 μM), but did shift the voltage-dependence of activation by ~−10 mV. We conclude that the M/V variation in IIIS5 and the S/A variation in the IIIS5-3P loop of Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 largely determine the difference in nifedipine potency between these two channels, but the difference in FPL potency is determined by divergent amino acids in the IIIS5-3P loop.

Introduction

Inhibitors of L-type voltage-gated Ca\textsuperscript{2+} channels have long been used in the treatment of cardiovascular diseases such as hypertension and angina pectoris. In these indications, the specific target is inhibition of Ca\textsubscript{v}1.2, the predominant L-type channel in vascular smooth muscle, to induce vasodilation (Catterall, 2000). However, the closely related L-type channel Ca\textsubscript{v}1.3 is expressed in SA and AV nodal tissue (Platzer et al., 2000), and is probably an important target for suppression of supraventricular arrhythmias. None of the three chemical classes of L-type channel blockers currently in clinical use [dihydropyridines (DHPs), phenylalkylamines (PAAs), or benzothiazepines (BTZs) (Hockerman et al., 1997b)] have a high degree of discrimination between Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3. Outside of the cardiovascular system, Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 are expressed in various types of neurons (Hell et al., 1993) and endocrine cells (Seino et al., 1992), where they are thought to play distinct roles in cellular regulation. For example, Ca\textsubscript{v}1.3 has been implicated in mediating Ca\textsuperscript{2+} oscillations in dopaminergic neurons of the substantia nigra that may lead to Ca\textsuperscript{2+} overload and contribute to the selective loss of these neurons in Parkinson's disease (Guzman et al., 2009, 2010; Surmeier and Schumacker, 2013). In addition, autoantibodies that activate Ca\textsubscript{v}1.3 have been detected in serum from patients with type 1 diabetes (Juntti-Berggren et al., 1993; Bason et al., 2013), suggesting a role for excessive Ca\textsubscript{v}1.3 activation in autoimmune-mediated β cell death. These observations have driven the search for selective inhibitors of Ca\textsubscript{v}1.3 as potential therapeutics for Parkinson disease and type 1 diabetes.

Given the attractiveness of Ca\textsubscript{v}1.3 as therapeutic targets, several efforts to develop subtype-selective L-type channel blockers have been published. One study examined dozens of derivatives of the DHP scaffold but reported only modest degrees of selectivity for Ca\textsubscript{v}1.3 over Ca\textsubscript{v}1.2 (Chang et al., 2010), and another study examining 5-unsubstituted DHPs reported compounds with better Ca\textsubscript{v}1.3 selectivity (Tenti et al., 2014). A screen of over 60,000 compounds identified a class of compounds, pyrimidine-2,4,6-triones, as moderately selective inhibitors of Ca\textsubscript{v}1.3 over Ca\textsubscript{v}1.2 (Kang et al., 2012, 2013). However, one follow-up study concluded that the selectivity of the lead pyrimidine-2,4,6-trione (compound 8) has been lost.

ABBRVIATIONS: Bay K 8644, methyl 2,6-dimethyl-5-nitro-4-[2-[trifluoromethyl]phenyl]-1,4-dihydropyridine-3-carboxylate; BTZ, benzothiazepines; DHP, dihydropyridines; FPL 64176, 2,5-dimethyl-4-[2-phenylmethyl]benzoyl]-1H-pyrole-3-carboxylic acid methyl ester; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; nifedipine, 1,4-dihydro-2,6-dimethyl-4-[2-nitrophenyl]-3,5-pyridinedicarboxylic acid dimethyl ester; NMDG, N-methyl-D-glucamine; PAA, phenylalkylamine.
was dependent on the subtype of the auxiliary β subunit expressed with Ca_{1.3} (Huang et al., 2014), and another concluded that compound 8 was an activator of L-type channels (Otnert et al., 2014).

The mixed results reported in studies using derivatives of DHPs or screens of chemical libraries suggest the need for more insight into differences between Ca_{1.2} and Ca_{1.3} that might be exploited in selective-drug development. The molecular pharmacology of Ca_{1.2} is well studied. The molecular determinants of Ca_{1.2} modulation by DHPs (Hockerman et al., 1997c; Sinnegger et al., 1997; Yamaguchi et al., 2003; Lin et al., 2011), PAAs (Hockerman et al., 1995, 1997a; Dilmac et al., 2004), and BtZs (Hering et al., 1996; Hockerman et al., 2000; Dilmac et al., 2003) have been identified, and homology models of the binding sites have been developed (Cosconati et al., 2007; Cheng et al., 2009; Tikhonov and Zhorov, 2009). On the other hand, the molecular pharmacology of Ca_{1.3} has not been extensively studied. One reason for this disparity may be that the critical residues for drug block of Ca_{1.2} are highly conserved in Ca_{1.3}, leading to the perception that the drug binding site in both channels is identical. However, Ca_{1.3} is reported to be less sensitive to block by some DHPs than Ca_{1.2} (Xu and Lipscombe, 2001; Huang et al., 2013), but the molecular determinants that mediate this difference in DHP affinity are not known.

The transmembrane domains of Ca_{1.2} and Ca_{1.3} that compose the drug binding pockets are nearly identical, but two subtle differences, one each in IIIS5 and IIIS6, exist. In the transmembrane I lSS-3P domains of these channels are highly divergent. The I lSS-3P domain contains two amino acid residues that are critical for DHP block of Ca_{1.2} (Yamaguchi et al., 2000, 2003), yet these residues are conserved between Ca_{1.2} and Ca_{1.3}. However, another cluster of amino acids, closer to I lSS5 and not conserved between Ca_{1.2} and Ca_{1.3}, is reported to influence DHP binding affinity (Wang et al., 2007). Therefore, we examined if substitution of these key divergent amino acids from Ca_{1.2} into Ca_{1.3} could reduce the IC_{50} for nifedipine (1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinecarboxylic acid dimethyl ester) and EC_{50} for the L-type channel agonist FPL 64176 compared with wild-type Ca_{1.3}.

Materials and Methods

Chemicals and Reagents. All reagents, unless otherwise indicated, were purchased from MilliporeSigma (St. Louis, MO). Oligonucleotides used for site-directed mutagenesis were obtained from GenScript (Nanjing, People’s Republic of China). The Ca_{1.3,2} (AF370010) and Ca_{1.3,2a} (AF370009) clones (Xu and Lipscombe, 2001) with three cloning errors repaired (Huang et al., 2013) were the gift of Dr. Tuk-Wah Soong, University of Singapore. The Ca_{1.2} clone (M67515) (Snotch et al., 1991) was the gift of Dr. Terrance Snotch, University of British Columbia.

Cell Culture. The tsA201 variant of the human embryonic kidney 293 cell line was grown at 37°C, 5% CO_{2}, in Dulbecco’s modified Eagle’s medium (Life Technologies/Thermo Fisher Scientific, Grand Island, NY) supplemented with 10% fetal bovine serum (Atlanta Biologic, Lawrenceville, GA), 100 IU/ml penicillin, and 100 μg/ml streptomycin.

Site-Directed Mutagenesis. All mutant Ca_{1.3,2a} (in pcDNA6) and Ca_{1.2} (in pcDNA3) α_{1} subunits (except Ca_{1.3} α_{1} + ) were constructed as described previously (Dilmac et al., 2003). To construct Ca_{1.3} α_{1} + , an oligonucleotide encoding amino acids 1058-1118 of Ca_{1.2} was ligated into Ca_{1.3,2a} in pSPORT6 after excision of the I lSS-5P encoding DNA with BamH1 and BstB1. The final version in Ca_{1.3,2a} pcDNA6 was created by ligation of the BamH1/EcoRV fragment containing the chimeric region from pSPORT6. All mutant constructs were verified by DNA sequencing and restriction digest analysis.

Electrophysiological Recordings. Mutant and wild type Ca_{1.2} or Ca_{1.3} α_{1} subunits were coexpressed with α_{1}β_{1} (Williams et al., 1993) and β_{1} (Castellano et al., 1993) subunits (both in pcDNA3), and pEgFPN1 (Clontech, Mountain View, CA) by transfection of tsA201 cells, as described previously (Dilmac et al., 2003). Transfected cells were identified by green fluorescent protein fluorescence. Micropipettes were pulled from borosilicate capillaries to an inside diameter of approximately 3–5 μm using a Sutter P-87 pipette puller (Sutter Instruments, Novato, CA), and polished with a Narishige MF 830 microforge (Narishige, Amityville, NY). The pipette solution contained: (in millimolars) 180 NMDG, 4 MgCl_{2}, 12 phosphocreatine, 5 BAPTA, 2 Na_{2}ATP, 0.5 Na_{3}GTP, 0.1 leupeptin, and pH was adjusted to 7.3. The extracellular solution contained (in millimolars): 140 NaCl, 20 CsCl_{2}, 10 BaCl_{2}, 10 HEPES, 10 glucose, 10 sucrose, 1 MgCl_{2}, and pH was adjusted to 7.4. In experiments with balanced NMDG, the extracellular solution was altered to contain 30 mM NMDG, with a corresponding reduction in NaCl concentration. Whole-cell voltage clamp recordings were made at room temperature using an Axopatch 200B amplifier (Axon Instrument, Sunnyvale, CA). Data were sampled at 10 kHz and filtered at 1 kHz. Drugs were applied in the extracellular solution with a Biologic RSC 160 perfusion system (Biologic, Sayssinet-Pariset, France). logIC_{50} values for nifedipine block were determined by fitting the fraction of current blocked at each drug concentration to the equation: Fraction Blocked = a – ((IC_{50}/IC_{50}))^{b}, where a = maximum fraction blocked, b = slope. logEC_{50} values for FPL potentiation were determined by normalizing the increase in current with each concentration of FPL to the increase in current observed with 10 μM FPL. When fitting equations to the nifedipine dose-response data (logIC_{50}), we set the minimum at zero, and let the slope and maximal block vary. This reflects the experimental observation that current block is often incomplete even at maximally effective concentrations. When fitting equations to the FPL 64176 dose-response data (logEC_{50}), we set the minimum at zero and the maximum at 1 (maximal current stimulation) but allowed slope to vary. The range of N values for dose-response curves represent the number of data points for each drug concentration. The number of separate experiments performed (i.e., cells clamped) to obtain a given dose-response curve is equal to or greater than the highest number of replicates indicated for any single drug concentration. The basis of the logIC_{50} and logEC_{50} values ± S.E.F. of the fit shown in Table 1 is the fit of all of the data for a given channel construct. V_{1/2} activation values were determined by plotting normalized tail-current amplitudes versus the corresponding 100-millisecond depolarizing voltage steps from −50 to +60 mV, in 10 mV-increments, from a holding potential of −80 mV. The data were fit to the equation, I = I_{1}(1 + exp((V_{1/2} - V)/k)), where k is a slope factor. The steady-state inactivation protocol used 10-second conditioning pulses from −80 to +20 mV in 10 mV-increments from a holding potential of −90 mV, followed by a 100-millisecond test pulse to +10 mV. V_{1/2} inactivation was determined by plotting the normalized test pulse amplitude versus the conditioning pulse potential, and fitting the data to the equation I = I_{1}(1 + exp(−(V – V_{1/2})/k)), where k is a slope factor. When fitting equations to the data for voltage-dependence of activation and inactivation, we set curves to start at 0 or 1, respectively, and force the curves to plateau at 1 or 0, respectively. Slopes were allowed to vary. The time course of channel deactivation was determined by fitting tail-current decay to either a single or double exponential function.

Homology Models of Ca_{1.2} and Ca_{1.3} on the Basis of the Structure of Ca_{1.1}. Homology models of Ca_{1.2} and Ca_{1.3} were generated using SWISS-MODEL (Guex et al., 2009; Benkert et al., 2011; Bertoni et al., 2017; Bienert et al., 2017; Waterhouse et al., 2018).
The structure of Ca1.1 (PDB-code: 5gw) was used as template for modeling (Wu et al., 2016). Ca1.2 and Ca1.3 share sequence identities of 72% and 71% with Ca1.1, respectively.

**Data Analysis and Statistics.** Data were analyzed using Clampfit 10.6 (Axon Instruments) and Sigmaplot 11 (Systat Software, San Jose, CA). logIC50 and logEC50 values were determined using GraphPad Prism 7.04 (GraphPad Software, La Jolla, CA). Comparisons of two or more means were made using one-way analysis of variance. Comparisons of three or more means were made using one-way analysis of variance. *P* < 0.05 was considered significant. Data shown are means ± S.E. Lines are fits of the equations indicated for each type of experiment to the data.

**Results**

**Characterization of Ba2+ Current Conducted by Ca1.2 or Ca1.3 Coexpressed with the β2 and α2δ1 Subunits in tsA201 Cells.** We assessed the biophysical and pharmacological properties of Ca1.2 and Ca1.3 in our expression system. As expected, Ca1.3 activated at more negative voltages than Ca1.2 (*P* < 0.001) (Fig. 1A; Table 1), and Ca1.2 inactivated at slightly more negative voltages than Ca1.3 (*P* < 0.01) (Fig. 1B; Table 1). We next examined the potency of nifedipine block of both channel types. We chose nifedipine because it is the most compact of the dihydropyridines, which revealed a substantial difference in nifedipine potency in blocking Ca1.2 compared with Ca1.3 (Supplemental Fig. 1). Channels were activated with 100-millisecond sample traces and the compiled dose-response curves for both perfusion capillary in the bath solution. Figure 1C shows sample traces and the compiled dose-response curves for both channel subtypes. As expected, Ca1.2 was blocked more potently by nifedipine than Ca1.3, with IC50 values of 22 ± 2 nM and 289 ± 30 nM, respectively (*P* < 0.001). The truncated splice variant Ca1.3/42a (Xu and Lipscombe, 2001) had been reported to be less sensitive to nifedipine than the full-length Ca1.3/42 variant (Huang et al., 2013). Therefore, we examined the dose-dependence of nifedipine block of Ca1.3/42a, and determined the IC50 for nifedipine to be 436 ± 24 nM, greater than that of Ca1.3/42 (*P* < 0.01) (Fig. 1C). We chose to use the full-length Ca1.3/42 variant in the subsequent experiments, since it is structurally more similar to the Ca1.2 variant used in this study.

### The IIIS5 Transmembrane Domain Plays a Key Role in Nifedipine Block of Ca1.3.

Studies in Ca1.2 have established transmembrane domain IIIS5 as a key component of the DHP binding pocket (Mitterdorfer et al., 1996). Specifically, mutations of T1039 and Q1043 (underlined in Fig. 2A) to the corresponding residues in DHP-insensitive voltage-gated Ca2+ channels results in a Cav1.2 mutant channel (termed Ca1.2/DHPi) that is markedly less sensitive to DHPs but normally sensitive to diltiazem (Hockerman et al., 2000; Lin et al., 2011). We made the corresponding Cav1.3/DHPi mutant, and as expected, it was substantially less sensitive to nifedipine than Ca1.3 (Fig. 2B). In fact, we were unable to determine the maximum percent of Ca1.3/DHPi current blocked because the nifedipine concentrations at the high end of the range (>200 μM) were at the limit of aqueous solubility (Ran et al., 2002). Assuming maximal inhibition of 90% of current, we estimated the IC50 of nifedipine block of Ca1.3/DHPi to be ~93 μM, more than 300 times that for Ca1.3. As with the corresponding mutation in Ca1.2 (Hockerman et al., 2000), the sensitivity of Ca1.3/DHPi block by the BTZ diltiazem was not reduced compared with Ca1.3 (Supplemental Fig. 2).

Given that transmembrane domain IIIS5 clearly contributes to the DHP binding pocket in Ca1.3, we next examined the single amino acid in this domain that is not conserved between Ca1.2 and Ca1.3, M1030 (Fig. 2A). The corresponding position...
in Cav1.2 (1036) is occupied by a V residue, so we constructed the mutant channel Cav1.3/MV, to determine if this conservative change could contribute to the difference in nifedipine potency between Cav1.2 and Cav1.3. The V1/2 inactivation of Cav1.3/MV was not different from that of Cav1.3; however, the V1/2 activation of Cav1.3/MV (-26 ± 1.1) (Table 1) was more positive than that for Cav1.3 (P < 0.05). The M1030V mutation increased the potency of nifedipine block of Cav1.3, reducing the IC50 from 289 to 89 ± 0.6 mV for Cav1.2 (N = 6) and -36 ± 1.3 mV (N = 5) for Cav1.3 (P < 0.001). We reasoned that this difference in P configuration could contribute to the difference in nifedipine potency, we next examined the role of the extracellular domain just downstream of IIIS5, the IIIS5-3P loop, and thus DHP affinity. Therefore, we created a chimeric channel, Cav1.3+/Cav1.2, that incorporates the Cav1.2 IIIS5-3P loop into the Cav1.3 background, to determine the effect of this region on the potency of nifedipine block. The voltage-dependence of activation and inactivation were both essentially unchanged in Cav1.3+/ compared with Cav1.3 (see Table 1). However, the IC50 for nifedipine block of Cav1.3+, (101 ± 4 nM) was reduced compared with that for Cav1.3 (P < 0.001) (Fig. 3B).

We next asked if a particular region of the IIIS5-3P loop could account for the increase in nifedipine potency in block of Cav1.3+ versus Cav1.3. The IIIS5-3P loop extends from the end of IIIS5 to the conserved E residue in the domain III selectivity filter (Fig. 3A). The region just upstream of the conserved selectivity filter E residue of homologous domain III (Yang et al., 1993) (1118 in Cav1.2, 1112 in Cav1.3; Fig. 3A) is known to be involved in DHP modulation of Cav1.2 (Yamaguchi et al., 2000, 2003) but is highly conserved between Cav1.2 and Cav1.3. Mutation of the nearest nonconserved residue upstream of E1112 in Cav1.3 (S1100) resulted in a channel (Cav1.3/SA) with V1/2 activation not different from Cav1.3 but with markedly left-shifted V1/2 inactivation (see Table 1). The IC50 for nifedipine block of Cav1.3/SA was 99 ± 24 nM (N = 6), indistinguishable from that for Cav1.3+ (Fig. 3B). We reasoned that this difference in P configuration could affect the conformation of the conserved, distal portion of the IIIS5-3P loop, and thus DHP affinity. Therefore, we created Cav1.3/PEEP, with P residues at position 1081 and 1085, but a P to E switch at position 1063, mimicking the P configuration of the Cav1.2 IIIS5-3P loop. The voltage-dependence of...
inactivation was not different from Ca\textsubscript{1.3}, and the voltage-dependence of activation was \(\sim 3\) mV more negative than Cav\textsubscript{1.3} (see Table 1). The IC\textsubscript{50} for nifedipine block of Cav\textsubscript{1.3}/PEEP was 188 \(\pm\) 28 nM, not statistically significantly lower than the IC\textsubscript{50} for block of Cav\textsubscript{1.3} (Fig. 3C; Table 1). However, the Hill slope for the dose-response curve for nifedipine block of Cav\textsubscript{1.3}/PEEP (0.43 \(\pm\) 0.02) was shallower than Cav\textsubscript{1.3} (\(P < 0.001\)). We next turned our attention to a region of the IIIS\textsubscript{5}-3P loop proximal to IIIS\textsubscript{5} that contains a cluster of three negatively charged residues in Cav\textsubscript{1.2} (D1063, E1069, E1071), reported to affect DHP binding affinity (Wang et al., 2007). Only two of these negative charges are conserved in Cav\textsubscript{1.3} (D1057 and E1065); moreover, the amino acid sequence surrounding these residues is highly divergent between Cav\textsubscript{1.2} and Cav\textsubscript{1.3} (see Fig. 3A). Therefore, we created the mutant Cav\textsubscript{1.3}/N6 with the Cav\textsubscript{1.2} sequence from amino acid 1064–1070 (SSKQTEA) inserted into the corresponding position (1058–1064) in Cav\textsubscript{1.3}. We found that expression of Cav\textsubscript{1.3}/N6 yielded functional channels, but the current was outward with 180 mM NMDG in the intracellular solution and no NMDG in the extracellular solution. Therefore, we used NMDG-balanced solutions in recordings with Cav\textsubscript{1.3}/N6, which restored inward barium current. The voltage-dependence of activation of Cav\textsubscript{1.3}/N6 with these conditions was \(-17 \pm 0.8\) mV, and the voltage-dependence of inactivation was \(-34 \pm 0.6\) mV (Table 1). We found that the IC\textsubscript{50} for nifedipine block of Cav\textsubscript{1.3}/N6 (116 \(\pm\) 53 nM) was slightly (\(P < 0.05\)) lower than that for Cav\textsubscript{1.3}, but the Hill slope of the dose-response curve (0.52 \(\pm\) 0.1) was also less than Cav\textsubscript{1.3} (\(P < 0.05\)) (Fig. 3D).

Given that the decreases in nifedipine IC\textsubscript{50} for both Cav\textsubscript{1.3}/MV and Cav\textsubscript{1.3}+ were relatively modest, we asked if combining these mutations would further increase the potency of nifedipine block. The V\textsubscript{1/2} activation of the resulting mutant channel, Cav\textsubscript{1.3}+V, was not different from Cav\textsubscript{1.3}, but the V\textsubscript{1/2} inactivation was shifted by \(-6\) mV (see Table 1). However, the IC\textsubscript{50} for nifedipine block of Cav\textsubscript{1.3}+V was reduced to 42 \(\pm\) 5 nM (\(N = 4–6\)), greater than the IC\textsubscript{50} for block of Cav\textsubscript{1.2} (\(P < 0.05\)). (E) Voltage-dependent activation of Cav\textsubscript{1.2}/VM. The V\textsubscript{1/2} activation for Cav\textsubscript{1.2}/VM was \(-24 \pm 1\) mV (\(N = 8\)), more negative than that for Cav\textsubscript{1.2} (\(P < 0.05\)).

We next asked if the small remaining gap in nifedipine potency between Cav\textsubscript{1.3}+V and Cav\textsubscript{1.2} could be closed. Besides IIIS\textsubscript{5} and the IIIS\textsubscript{5}-3P loop, transmembrane domain IIIS\textsubscript{6} also contributes to the DHP binding pocket in Cav\textsubscript{1.2} (Hockerman et al., 1997b). The only amino acid residue in IIIS\textsubscript{6} not conserved between Cav\textsubscript{1.2} and Cav\textsubscript{1.3} is an I/V divergence at position 1156/1150; moreover, mutation of I1156 in Cav\textsubscript{1.2} to A resulted in a significant decrease in DHP binding affinity (Peterson et al., 1997). Unfortunately, we found that substitution of V for I at position 1150 in IIIS\textsubscript{6} of either Cav\textsubscript{1.3}+V or Cav\textsubscript{1.3}/MV resulted in channels that yielded little to no current upon expression in tsA201 cells.
Differences in the IIIS5-3P Loop Are Responsible for the Difference in Potency of FPL in Ca,1.2 and Ca,1.3. The nondihydropyridine compound FPL 64176 (FPL) (Ginap et al., 1993) is a well characterized potentiator of Ca,1.2 current (Liu et al., 2003). Reconstruction of the DHP binding site in the P/Q-type channel Ca,2.1 conferred potentiation of current by FPL, as well as potent block by DHP antagonists (Sinnegger et al., 1997). However, very little is known about FPL modulation of Ca,1.3. Therefore, we compared the potency of FPL potentiation of current in Ca,1.2 and Ca,1.3. The experiments with Ca,1.3 used balanced NMDG solutions because we found that application of FPL frequently induced outward current when the extracellular IIIS5-3P loop contained no NMDG (Supplemental Fig. 3A), suggesting that FPL binding substantially affects the permeability of Ca,1.3 to NMDG. We found that the EC50 for potentiation of current amplitude in Ca,1.2 by FPL was 103 ± 40 nM (Fig. 4, A and D). In contrast, the EC50 for potentiation of Ca,1.3 current amplitude by FPL was 854 ± 236 nM (P < 0.05) (Fig. 4, B and D). Thus, as with nifedipine, Ca,1.3 is less sensitive to FPL than Ca,1.2.

We next asked if some of the same differences between Ca,1.2 and Ca,1.3 that account for the difference in nifedipine potency could also account for the difference in the potency of FPL in these two channel subtypes. We first measured the potency of FPL potentiation of current in Ca,1.3+V, since this mutant had nearly the same sensitivity to nifedipine as Ca,1.2. We were able to perform these experiments in the standard solution set, since FPL did not induce outward current in Ca,1.3+V. The EC50 for potentiation of current amplitude by FPL in Ca,1.3+V was 99 ± 5 nM (Fig. 4, C and D), indistinguishable from the EC50 of FPL for potentiation of Ca,1.2. We measured the EC50 for FPL potentiation of Ca,1.3/MV current amplitude in the standard solution set since we did not observe outward currents in the presence of FPL in this mutant. The EC50 of FPL for Ca,1.3/MV was 737 ± 20 nM, not different from the EC50 for Ca,1.3 (Fig. 4D). Taken together, these results suggest that the molecular determinants of the difference in potency of FPL lie within the IIIS5-3P loop. Ca,1.3+V exhibited outward current in the presence of FPLs, similar to Ca,1.3 (Supplemental Fig. 3A). However, we were unable to measure the potency of FPL potentiation of this mutant because, even in the NMDG-balanced solution set, FPL induced erratic changes in current amplitude (Supplemental Fig. 3B). We were able to measure the potency of FPL potentiation of the IIIS5-3P loop mutants Ca,1.3/PEEP, Ca,1.3/N6, and Ca,1.3/SA, and found that none of these mutants displayed increased sensitivity to potentiation of current by FPL compared with Ca,1.3 (Table 1). Thus, we have identified two regions of amino acid divergence between Ca,1.2 and Ca,1.3 within the IIIS5-3P loop, Ca,1.2 1106/Ca,1.3 1100 and Ca,1.2 1064–1070/Ca,1.3 1058–64, that...
appear to confer differences in sensitivity to nifedipine block but not in FPL potentiation of these two channels.

FPL has a strong effect on the kinetics of deactivation as well as the voltage-dependence of activation of Ca\(_{\text{v}}\)1.2 and Ca\(_{\text{v}}\)1.3. Figure 5 shows the effect of 10 \(\mu\)M FPL on tail-current kinetics, a measure of the rate of deactivation. Ca\(_{\text{v}}\)1.3 displays a fast rate of closing with a single time constant (\(\tau\)) in the absence of FPL, but a second, slower \(\tau\) is observed in the presence of FPL (Fig. 5A; Table 2). In contrast, deactivation in Ca\(_{\text{v}}\)1.2 in the absence of FPL follows two \(\tau\)s. However, a single slow \(\tau\) is principally observed in the presence of FPL that is greater than both \(\tau\)s in the absence of FPL (Fig. 5B; Table 2).

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**Fig. 4.** Potency of FPL 64176 potentiation of Ca\(_{\text{v}}\)1.2, Ca\(_{\text{v}}\)1.3, and mutant channels. (A–C) Example traces showing FPL potentiation of Ca\(_{\text{v}}\)1.2, Ca\(_{\text{v}}\)1.3, and Ca\(_{\text{v}}\)1.3 FPL, respectively. Note the marked slowing of the tail current in Ca\(_{\text{v}}\)1.2 that is absent in Ca\(_{\text{v}}\)1.3. (D) Dose-response curves for FPL 64176 potentiation of Ca\(_{\text{v}}\)1.2, Ca\(_{\text{v}}\)1.3, and mutant channels. The \(EC_{50}\) values for FPL potentiation of current for Ca\(_{\text{v}}\)1.2 and Ca\(_{\text{v}}\)1.3 were 103 ± 40 nM (\(N = 3\)–\(7\)) and 854 ± 236 nM (\(N = 3\)–\(7\)), respectively (\(P < 0.05\)). The \(EC_{50}\) for FPL potentiation of the mutant Ca\(_{\text{v}}\)1.3+V (99 ± 5 nM) (\(N = 3\)–\(7\)) was not different from that of Ca\(_{\text{v}}\)1.2 but was different from that of Ca\(_{\text{v}}\)1.3 (\(P < 0.05\)). In contrast, the \(EC_{50}\) for FPL potentiation of the mutant Ca\(_{\text{v}}\)1.3/MV was 737 ± 20 nM (\(N = 5\)), not different from that of Ca\(_{\text{v}}\)1.3. Data are shown as the mean fractional increase in current compared with 10 \(\mu\)M FPL 64176 ± S.E.

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**Fig. 5.** Kinetics of tail-current decay in the presence and absence of FPL 64176 in Ca\(_{\text{v}}\)1.2, Ca\(_{\text{v}}\)1.3, and mutant channels. (A) Example 100-millisecond depolarization demonstrating tail-current decay in Ca\(_{\text{v}}\)1.3 in the presence or absence of 10 \(\mu\)M FPL 64176. (B) Example 100-millisecond depolarization demonstrating tail-current decay in Ca\(_{\text{v}}\)1.2 in the presence or absence of 10 \(\mu\)M FPL 64176. (C) The R10 value (fraction of tail current remaining 10 milliseconds after peak) in the presence of FPL was greater in Ca\(_{\text{v}}\)1.2 (0.67 ± 0.09, \(N = 6\)) compared with that of Ca\(_{\text{v}}\)1.3 (0.15 ± 0.02) (\(N = 5\)) (***\(P < 0.001\)). (D) Example 100-millisecond depolarization demonstrating tail-current decay in Ca\(_{\text{v}}\)1.3/DHPi in the presence or absence of 10 \(\mu\)M FPL 64176. (E) The time constant for deactivation of Ca\(_{\text{v}}\)1.3/DHPi (\(\tau = 0.59 ± 0.11\) milliseconds, \(N = 5\)) was not affected by the presence of 10 \(\mu\)M FPL (\(\tau = 0.60 ± 0.04\) milliseconds, \(N = 5\)). (F) Example 100-millisecond depolarization demonstrating tail-current decay in Ca\(_{\text{v}}\)1.3/MV in the presence or absence of 10 \(\mu\)M FPL 64176. (G) The time constant for deactivation of Ca\(_{\text{v}}\)1.3/MV in the presence of FPL followed a single time constant (\(\tau = 0.70 ± 0.13\) milliseconds, \(N = 5\)) that was slower than that of Ca\(_{\text{v}}\)1.3 (**\(P < 0.01\)). The R10 value for Ca\(_{\text{v}}\)1.3/MV tail current in the presence of 10 \(\mu\)M FPL (0.51 ± 0.15, \(N = 5\)) was greater than that of Ca\(_{\text{v}}\)1.3 (*\(P < 0.05\)).
Given the differences in the kinetics of deactivation in Cav1.3 versus Cav1.2, we compared the FPL-induced slowing of deactivation in these channels by measuring the fraction of the tail current remaining 10 milliseconds after reaching peak (R10). The R10 for both Cav1.2 and Cav1.3 in the absence of FPL was negligible. Figure 5C shows that the R10 of Cav1.2 in the presence of 10 μM FPL (0.67 ± 0.09) was greater than that of Cav1.3 (0.15 ± 0.02) (P < 0.001), indicating a slower slowing of deactivation by FPL in Cav1.2. We also found that FPL shifted the V1/2 activation of Cav1.2 by −7 mV in Cav1.3 (Table 2). Thus, FPL is not only more potent in stimulating current amplitude in Cav1.2 compared with Cav1.3 but also has stronger effects on deactivation kinetics and the voltage-dependence of activation in Cav1.2 at a maximally effective concentration (10 μM).

We next asked if the Cav1.3/DHPi channel was less sensitive to FPL than Cav1.3. Deactivation of Cav1.3/DHPi followed a single τ that was not different from that of Cav1.3 (Table 2) but was not altered by 10 μM FPL (Fig. 5, D and E). Not surprisingly, no significant increase in current was observed upon application of 10 μM FPL to Cav1.3/DHPi. Interestingly, 10 μM FPL did shift the V1/2 activation of Cav1.3/DHPi by −9 mV (Table 2). Since IIIS5 is clearly crucial for the action of FPL, we examined the kinetics of deactivation in Cav1.3/MV (Fig. 5F). In the absence of FPL, Cav1.3/MV deactivation followed a single τ (0.71 ± 0.04 milliseconds) that was slightly, but statistically significantly greater than Cav1.3 (0.41 ± 0.07 milliseconds) (P < 0.01) (Fig. 5G). In the presence of 10 μM FPL, the R10 was greater in Cav1.3/MV (0.52 ± 0.15 milliseconds) (P < 0.05) compared with Cav1.3 (Fig. 5H).

Table 2

<table>
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<tr>
<th>Channel</th>
<th>Frac. Fast</th>
<th>τ-Fast (ms)</th>
<th>Frac Slow</th>
<th>τ-Slow</th>
<th>Frac Slow FPL</th>
<th>τ-FPL</th>
<th>FPL R10</th>
<th>N</th>
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<tr>
<td>Cav1.2</td>
<td>0.79 ± 0.08</td>
<td>0.37 ± 0.05</td>
<td>0.18 ± 0.08</td>
<td>6.5 ± 0.6</td>
<td>0.64 ± 0.20</td>
<td>24 ± 7#</td>
<td>0.67 ± 0.09**</td>
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</tr>
<tr>
<td>Cav1.3</td>
<td>0.96 ± 0.01</td>
<td>0.41 ± 0.07</td>
<td>NA</td>
<td>NA</td>
<td>0.34 ± 0.06</td>
<td>11 ± 1##</td>
<td>0.15 ± 0.02</td>
<td>5</td>
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<tr>
<td>Cav1.2/DHPi</td>
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<td>0.59 ± 0.11</td>
<td>NA</td>
<td>NA</td>
<td>0.60 ± 0.04</td>
<td>ND</td>
<td>5</td>
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<tr>
<td>Cav1.3+V</td>
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<td>0.70 ± 0.13</td>
<td>NA</td>
<td>NA</td>
<td>0.84 ± 0.05</td>
<td>6.0 ± 1##</td>
<td>0.39 ± 0.07*</td>
<td>6</td>
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<tr>
<td>Cav1.3/MV</td>
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<td>0.71 ± 0.04*</td>
<td>NA</td>
<td>NA</td>
<td>0.53 ± 0.13</td>
<td>39 ± 9###</td>
<td>0.52 ± 0.15*</td>
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<tr>
<td>Cav1.2/VM</td>
<td>0.96 ± 0.04</td>
<td>0.68 ± 0.15</td>
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<td>NA</td>
<td>0.73 ± 0.10</td>
<td>29 ± 7##</td>
<td>0.71 ± 0.08***</td>
<td>6</td>
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<tr>
<td>Cav1.3/PEEP</td>
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<td>0.77 ± 0.08**</td>
<td>NA</td>
<td>NA</td>
<td>0.71 ± 0.18</td>
<td>12 ± 3##</td>
<td>0.34 ± 0.11</td>
<td>5</td>
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</table>

#P < 0.05; **P < 0.01; ***P < 0.001 compared with Cav1.3.
##P < 0.05; ###P < 0.01; *P < 0.001 compared with absence of FPL.

Discussion

The voltage-dependence and sensitivity to nimodipine (a DHP antagonist) of the Ca1.3 cDNA used in this study (Ca1.342) was previously characterized (Xu and Lipscombe, 2001). The V1/2 activation reported here is indistinguishable from that initial characterization. Further, Xu and Lipscombe reported an ~20-fold higher IC50 for nimodipine block of Cav1.3 compared with Cav1.2. For our comparison of DHP antagonist potency, we chose nimodipine since it is the most compact molecule in this class, with no extended side chains that might interact with amino acids outside of the canonical DHP binding site, yet it retains excellent potency. Our results indicating an ~13-fold higher IC50 for nifedipine block of Cav1.3 compared with Cav1.2 is in line with the decreased potency of nimodipine in block of Cav1.3 compared with Cav1.2 reported by Xu and Lipscombe. Though they did not report an EC50 for agonist potentiation of Cav1.3, Xu and Lipscombe did report a modest shift in V1/2 activation of Cav1.3 by 1 μM concentration of DHP agonist Bay K 8644 (−7 mV), similar to the modest leftward shift in V1/2 activation we observed in Cav1.3 in the presence of 10 μM FPL. Thus, our data show that our expression system recapitulates the primary differences between Cav1.2 and Cav1.3, most notably, the left-shifted activation and lower sensitivity of current to block by DHP antagonists of Cav1.3 compared with Cav1.2.

Another study examined both the binding affinity and block potency of the DHP antagonist PN200-110 (isradipine) for a Ca1.3 clone from human pancreas (Ca1.3α) (Koschak et al., 2001). Interestingly, the Kp for [3H]PN200-110 binding was not significantly different between Ca1.3α and Cav1.2 cloned from rabbit cardiac muscle (Tanabe et al., 1987). However, the IC50 for block of current by PN200-110 was reported to be 8.5-fold higher for Ca1.3α than Cav1.2, in excellent agreement with the difference in nifedipine potency in blocking Ca1.3 and Cav1.2 in this study. Kp values for binding of DHPs to L-type channels in isolated membranes are invariably lower than IC50 values for current block. For example, the Kp for binding of [3H]PN200-110 to the Ca1.2 clone used in this study is 55 pM, whereas the IC50 for PN200-110 block is 7 nM (Peterson et al., 1997). Binding isotherms in both studies clearly indicated a single [3H]PN200-110 binding site, which probably reflects the open, inactivated state of the channel at 0 mV. Thus, it is probable that the Hill slopes different from 1 that we observed for nifedipine block of Ca1.3 and some of the mutant channels used in this study reflect the presence of distinct voltage-dependent channel conformations that regulate DHP affinity.

Though the DHP binding pockets of Ca1.2 and Ca1.3 are highly conserved, our results suggest that relatively minor differences in transmembrane segment IIIS5 and the IIIS5-3P loop can largely account for the difference in potency of nifedipine in block of Cav1.2 and Cav1.3. The IIIS5 helix is clearly a critical component of the Ca1.3 DHP binding pocket, as mutation of T1033 and Q1037 in Cav1.3/DHPi results in a marked loss of nifedipine potency. The side chains of M1030/V1036 in Ca1.3 and Ca1.2 are projected to align to the same face of the IIIS5 helix as the T and Q residues required for high-potency DHP block (Mitterdorfer et al., 1996), supporting our finding that swapping the Ca1.3-specific residue at M1030/V1036 in Cav1.3 and Cav1.2 results in a greater potency of FPL block. Additionally, our observation that swapping the Cav1.3-specific residue at IIIS5 helix can largely account for the difference in potency of nifedipine in block of Cav1.3 and Cav1.2, which probably reflects the open, inactivated state of the channel at 0 mV. Thus, it is probable that the Hill slopes different from 1 that we observed for nifedipine block of Ca1.3 and some of the mutant channels used in this study reflect the presence of distinct voltage-dependent channel conformations that regulate DHP affinity.

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this position into Ca\textsubscript{1.2} (V1036M) shifts nifedipine potency toward that of Ca\textsubscript{1.3}, and vice versa. Interestingly, the swap of channel subtype-specific residues in this position also results in small reciprocal shifts in $V_{1/2}$ activation (Table 1). However, only the Ca\textsubscript{1.3}/MV mutant exhibited slower deactivation, both in the presence and absence of FPL (Table 2). This observation, that decreasing the bulk of the amino acid side-chain at position 1030 in Ca\textsubscript{1.3} affects voltage-dependence of activation and the rate of tail-current decay, suggests that position 1030 in IIIS5 (outer pore helix) may interact with IIIS6 (inner pore helix) in a manner that regulates channel gating. Previously published models of DHP binding in Ca\textsubscript{1.2} suggest that amino acid residues directly interacting with DHP drugs are conserved between Ca\textsubscript{1.2} and Ca\textsubscript{1.3} (Cosconati et al., 2007; Tikhonov and Zhorov, 2009). To understand how subtle differences in amino acid sequence might account for a significant difference in nifedipine potency, we constructed homology models of Ca\textsubscript{1.3} and Ca\textsubscript{1.2} (Fig. 6) on the basis of the recently published high-resolution cryo-EM structure of Ca\textsubscript{1.1} (Wu et al., 2016). The models suggest that the increase in side-chain bulk between Ca\textsubscript{1.3} and Ca\textsubscript{1.2} at position 1030/1036 (M vs. V) could potentially decrease accessibility of nifedipine to the critical Q1037 and F1106 residues (Fig. 6A). In addition, the model predicts that S1100 in Ca\textsubscript{1.3} can form a hydrogen bond with N1094, an interaction that could potentially constrain the movement of the 3P helix during nifedipine binding (Fig. 6B). The corresponding positions in Ca\textsubscript{1.2} are occupied by an alanine residue (1106) and a glutamate (1100), precluding such an interaction (Fig. 6C). Interestingly, S1100 of Ca\textsubscript{1.3} is conserved in the corresponding position of Ca\textsubscript{1.1} (S1002) and the position corresponding to N1094 of Ca\textsubscript{1.3} is a histidine in Ca\textsubscript{1.1} (H996). These residues, with the assistance of D998, may form a hydrogen bond in Ca\textsubscript{1.1} (Fig. 6D), which may contribute to the lower binding affinity of Ca\textsubscript{1.1} for $[^3]$H]PN200-110 (270 pM) (Peterson et al., 1996) compared with Ca\textsubscript{1.2} (55 pM) (Peterson et al., 1997). Thus, our model suggests that the effect of the Ca\textsubscript{1.3}/A mutation on nifedipine potency is indirect, and that the displacement of the 3P helix may be required for high potency block of Ca\textsubscript{1.2} by DHP drugs.

Our studies of FPL potentiation of Ca\textsubscript{1.2}, Ca\textsubscript{1.3}, and the various mutant channels also yielded some novel results. First, Fig. 4 clearly shows that FPL is much more potent in potentiating current conducted by Ca\textsubscript{1.2} compared with Ca\textsubscript{1.3}. This difference can be ascribed completely to amino acid differences in the IIIS5-3P loop between these two channels. Nevertheless, the conserved T and Q residues in IIIS5 are clearly important for FPL action on Ca\textsubscript{1.3} even though the nearby M1030V mutation did not increase the potency of FPL action in isolation. However, the inclusion of V1030 in Ca\textsubscript{1.3}+V was critical for stabilizing FPL potentiation of current and revealing the increased sensitivity of this mutant to FPL. Interestingly, despite a complete loss of slowing of deactivation by FPL, the FPL-induced shift in $V_{1/2}$ activation in Ca\textsubscript{1.3}/DHPi was not different from that of Ca\textsubscript{1.3}, suggesting distinct sites of action on Ca\textsubscript{1.3} for these two characteristic effects of FPL on L-type channel gating. Unfortunately, we were not able to further resolve the amino acid residues that confer the difference in sensitivity to FPL between Ca\textsubscript{1.2} and Ca\textsubscript{1.3} beyond the IIIS5-3P loop, as none of the mutations within this domain that increased nifedipine potency improved FPL potency at Cav1.3. It is possible that these determinants may be among the sixteen other amino acid differences between Ca\textsubscript{1.2} and Ca\textsubscript{1.3} within this domain that we did not examine.

In our studies of the Ca\textsubscript{1.3}/N6 mutant, we made the unexpected observation that outward current often developed during the course of an experiment. The standard solution set used in this study sets up a large NMDG gradient across the membrane. Mutations in the pore region of Ca\textsubscript{1.2} were previously reported to lead to enhanced permeability of NMDG, as evidenced by a marked shift in reversal potential that was abolished by equalizing the NMDG concentration in the extracellular and intracellular solutions (Hockerman et al., 2016). The models suggest that the increase in side-chain bulk between Cav1.3 and Cav1.2 at position 1030/1036 (M vs. V) could potentially decrease accessibility of nifedipine to the critical Q1037 and F1106 residues (Fig. 6A). In addition, the model predicts that S1100 in Cav1.3 can form a hydrogen bond with N1094, an interaction that could potentially constrain the movement of the 3P helix during nifedipine binding (Fig. 6B). The corresponding positions in Cav1.2 are occupied by an alanine residue (1106) and a glutamate (1100), precluding such an interaction (Fig. 6C). Interestingly, S1100 of Cav1.3 is conserved in the corresponding position of Cav1.1 (S1002) and the position corresponding to N1094 of Cav1.3 is a histidine in Cav1.1 (H996). These residues, with the assistance of D998, may form a hydrogen bond in Cav1.1 (Fig. 6D), which may contribute to the lower binding affinity of Cav1.1 for $[^3]$H]PN200-110 (270 pM) (Peterson et al., 1996) compared with Cav1.2 (55 pM) (Peterson et al., 1997). Thus, our model suggests that the effect of the Cav1.3/S/A mutation on nifedipine potency is indirect, and that the displacement of the 3P helix may be required for high potency block of Cav1.2 by DHP drugs.

Our studies of FPL potentiation of Cav1.2, Cav1.3, and the various mutant channels also yielded some novel results. First, Fig. 4 clearly shows that FPL is much more potent in potentiating current conducted by Cav1.2 compared with Cav1.3. This difference can be ascribed completely to amino acid differences in the IIIS5-3P loop between these two channels. Nevertheless, the conserved T and Q residues in IIIS5 are clearly important for FPL action on Cav1.3 even though the nearby M1030V mutation did not increase the potency of FPL action in isolation. However, the inclusion of V1030 in Cav1.3+V was critical for stabilizing FPL potentiation of current and revealing the increased sensitivity of this mutant to FPL. Interestingly, despite a complete loss of slowing of deactivation by FPL, the FPL-induced shift in $V_{1/2}$ activation in Cav1.3/DHPi was not different from that of Cav1.3, suggesting distinct sites of action on Cav1.3 for these two characteristic effects of FPL on L-type channel gating. Unfortunately, we were not able to further resolve the amino acid residues that confer the difference in sensitivity to FPL between Cav1.2 and Cav1.3 beyond the IIIS5-3P loop, as none of the mutations within this domain that increased nifedipine potency improved FPL potency at Cav1.3. It is possible that these determinants may be among the sixteen other amino acid differences between Cav1.2 and Cav1.3 within this domain that we did not examine.
Interestingly, neither the Ca v1.3 Cav1.3 subunits, respectively. to alter the permeability of Cav1.2 (Fan et al., 2001), such that may play a role in the observed permeability changes in mutant conducted outward current in the presence of FPL in notion that FPL binding may induce conformational changes were unable to determine an EC50 for FPL stimulation of this divergence in transmembrane domain IIIS5, and an S/A tal Fig. 3A) that was abolished in Cav1.3 by equalizing the undergo current reversal upon FPL application (Supplemen-


Acknowledgments

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Authorship Contributions

Participated in research design: Wang, Hockerman.

Conducted experiments: Wang, Tang, Harvey, Hockerman.

Contributed new reagents or analytic tools: Salyer, Li, Rantz, Lill.

Performed data analysis: Wang, Tang, Harvey, Hockerman.

Wrote or contributed to the writing of the manuscript: Wang, Hockerman.

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