

**Molecular determinants of the differential modulation of Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3 by nifedipine and FPL  
64176**

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FPL 64176 (2,5-Dimethyl-4-[2-(phenylmethyl)benzoyl]-1*H*-pyrrole-3-carboxylic acid methyl ester); nifedipine (1,4-Dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinecarboxylic acid dimethyl ester); NMDG (N-methyl-D-glucamine); HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid); BAPTA (1,2-Bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid); Bay K 8644 (Methyl 2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]-1,4-dihydropyridine-3-carboxylate)

## Abstract

Nifedipine and FPL 64176 (FPL), which block and potentiate L-type voltage-gated Ca<sup>2+</sup> channels respectively, more potently modulate Ca<sub>v</sub>1.2 than Ca<sub>v</sub>1.3. To identify potential strategies for developing subtype-selective inhibitors, we investigated the role of divergent amino acid residues in transmembrane domains IIS5 and the extracellular IIS5-3P loop region in modulation of these channels by nifedipine and FPL. Insertion of the extracellular IIS5-3P loop from Ca<sub>v</sub>1.2 into Ca<sub>v</sub>1.3 (Ca<sub>v</sub>1.3+) reduced the IC<sub>50</sub> of nifedipine from 289 nM to 101 nM, and substitution of S1100 with an A residue, as in Ca<sub>v</sub>1.2, accounted for this difference. Substituting M1030 in IIS5 to V in Ca<sub>v</sub>1.3+ (Ca<sub>v</sub>1.3+V) further reduced the IC<sub>50</sub> of nifedipine to 42 nM. FPL increased current amplitude with an EC<sub>50</sub> of 854 nM in Ca<sub>v</sub>1.3, 103 nM in Ca<sub>v</sub>1.2, and 99 nM in Ca<sub>v</sub>1.3+V. In contrast to nifedipine block, substitution of M1030 to V in Ca<sub>v</sub>1.3 had no effect on potency of FPL potentiation of current amplitude, but slowed deactivation in the presence and absence of 10 μM FPL. FPL had no effect on deactivation of Ca<sub>v</sub>1.3/DHPi, a channel with very low sensitivity to nifedipine block (IC<sub>50</sub> ~ 93 μM), but did shift the voltage-dependence of activation by ~-10 mV. We conclude that the M/V variation in IIS5 and the S/A variation in the IIS5-3P loop of Ca<sub>v</sub>1.2 and Ca<sub>v</sub>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 IIS5-3P loop.

## Introduction

Inhibitors of L-type voltage-gated  $\text{Ca}^{2+}$  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  $\text{Ca}_v1.2$ , the predominant L-type channel in vascular smooth muscle, to induce vasodilation (Catterall, 2000). However, the closely related L-type channel  $\text{Ca}_v1.3$  is expressed in SA and AV nodal tissue (Platzer et al., 2000), and is likely an important target for suppression of supraventricular arrhythmias. None of the three chemical classes of L-type channels blockers (dihydropyridines (DHPs), phenylalkylamines (PAAs) or benzothiazepines (BTZs) (Hockerman et al., 1997b)) currently in clinical use have a high degree of discrimination between  $\text{Ca}_v1.2$  and  $\text{Ca}_v1.3$ . Outside of the cardiovascular system,  $\text{Ca}_v1.2$  and  $\text{Ca}_v1.3$  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,  $\text{Ca}_v1.3$  has been implicated in mediating  $\text{Ca}^{2+}$  oscillations in dopaminergic neurons of the substantia nigra that may lead to  $\text{Ca}^{2+}$  overload and contribute to the selective loss of these neurons in Parkinson's disease (Guzman et al., 2009; Guzman et al., 2010; Surmeier and Schumacker, 2013). In addition, autoantibodies that activate  $\text{Ca}_v1.3$  have been detected in serum from patients with Type 1 diabetes (Bason et al., 2013; Juntti-Berggren et al., 1993), suggesting a role for excessive  $\text{Ca}_v1.3$  activation in autoimmune mediated beta cell death. These observations have driven the search for selective inhibitors of  $\text{Ca}_v1.3$  as potential therapeutics for Parkinson's Disease and Type 1 diabetes.

Given the attractiveness of  $\text{Ca}_v1.3$  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  $\text{Ca}_v1.3$  over  $\text{Ca}_v1.2$  (Chang et al., 2010), while another study examining 5-unsubstituted DHPs reported compounds with better  $\text{Ca}_v1.3$  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  $\text{Ca}_v1.3$  over  $\text{Ca}_v1.2$  (Kang et al., 2012; Kang et al., 2013). However, one follow-up study concluded that the selectivity of the lead pyrimidine-2,4,6-trione (compound 8) was

dependent on the subtype of the auxiliary beta subunit expressed with Ca<sub>v</sub>1.3 (Huang et al., 2014), while another concluded that compound 8 was an activator of L-type channels (Ortner 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<sub>v</sub>1.2 and Ca<sub>v</sub>1.3 that might be exploited in selective drug development. The molecular pharmacology of Ca<sub>v</sub>1.2 is well studied. The molecular determinants of Ca<sub>v</sub>1.2 modulation by DHPs (Hockerman et al., 1997c; Lin et al., 2011; Sinnegger et al., 1997; Yamaguchi et al., 2003), PAAs (Dilmac et al., 2004; Hockerman et al., 1997a; Hockerman et al., 1995), and BTZs (Dilmac et al., 2003; Hering et al., 1996; Hockerman et al., 2000) have been identified, and homology models of the binding sites have been developed (Cheng et al., 2009; Cosconati et al., 2007; Tikhonov and Zhorov, 2009). On the other hand, the molecular pharmacology of Ca<sub>v</sub>1.3 has not been extensively studied. One reason for this disparity may be that the critical residues for drug block of Ca<sub>v</sub>1.2 are highly conserved in Ca<sub>v</sub>1.3, leading to the perception that the drug binding site in both channels is identical. However, Ca<sub>v</sub>1.3 is reported to be less sensitive to block by some DHPs than Ca<sub>v</sub>1.2 (Huang et al., 2013; Xu and Lipscombe, 2001), but the molecular determinants that mediate this difference in DHP affinity are not known.

The transmembrane domains of Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3 that comprise the drug binding pockets are nearly identical, but two subtle differences, one each in IIS5 and IIS6, exist. In addition, the extracellular IIS5-3P domains of these channels are highly divergent. The IIS5-3P domain contains two amino acid residues that are critical for DHP block of Ca<sub>v</sub>1.2 (Yamaguchi et al., 2000; Yamaguchi et al., 2003), yet these residues are conserved between Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3. However, another cluster of amino acids, closer to IIS5 and not conserved between Ca<sub>v</sub>1.2 and Ca<sub>v</sub>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<sub>v</sub>1.2 into Ca<sub>v</sub>1.3 could reduce the IC<sub>50</sub> for nifedipine and EC<sub>50</sub> for the L-type channel agonist FPL 64176 compared to wild type Ca<sub>v</sub>1.3.

## Materials and Methods

*Chemicals and Reagents*- All reagents, unless otherwise indicated, were purchased from Sigma-Aldrich (St. Louis, MO). Oligonucleotides used for site-directed mutagenesis were obtained from GenScript (Nanjing, PRC). The  $Ca_v1.3_{42}$  (AF370010) and  $Ca_v1.3_{42a}$  (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  $Ca_v1.2$  clone (M67515) (Snutch et al., 1991) was the gift of Dr. Terrance Snutch, University of British Columbia.

*Cell Culture*- The tsA201 variant of the HEK 293 cell line was grown at 37°C, 5% CO<sub>2</sub> in Dulbecco's Modified Eagle's Medium (Life Technologies, Grand Island, NY) supplemented with 10% Fetal Bovine Serum (Atlanta Biological, Lawrenceville, GA), 100 U/ml penicillin, and 100 µg/ml streptomycin.

*Site-directed Mutagenesis*- All mutant  $Ca_v1.3_{42}$  (in pcDNA6) and  $Ca_v1.2$  (in pcDNA3)  $\alpha_1$  subunits (except  $Ca_v1.3+$ ), were constructed as described previously (Dilmac et al., 2003). To construct  $Ca_v1.3+$ , an oligonucleotide encoding amino acids 1058-1118 of  $Ca_v1.2$  was ligated into  $Ca_v1.3_{42}$  in pSPORT6 after excising the IIS5-3P encoding DNA with BamH1 and BstB1. The final version in  $Ca_v1.3_{42}$  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  $Ca_v1.2$  or  $Ca_v1.3$   $\alpha_1$  subunits were co-expressed with  $\alpha_2\delta_1$  (Williams et al., 1992) and  $\beta_3$  (Castellano et al., 1993) subunits (both in pcDNA3), and pEGFPN1 (Clontech, Mountain View, CA) by transfection of tsA 201 cells, as described previously (Dilmac et al., 2003). Transfected cells were identified by GFP fluorescence. Micropipettes were pulled from borosilicate capillaries to an inside diameter of approximately 3-5 microns using a Sutter P-87 pipette puller (Sutter Instruments, Novato, CA), and polished with a Narishige MF 830 micro forge (Narishige, Amityville, NY). The pipette solution contained: (in mM) 180 NMDG, 40 HEPES, 4 MgCl<sub>2</sub>, 12 phosphocreatine, 5 BAPTA, 2 Na<sub>2</sub>ATP, 0.5 Na<sub>3</sub>GTP, 0.1 leupeptin, and pH was adjusted to 7.3. The extracellular solution

contained (in mM): 140 NaCl, 20 CsCl<sub>2</sub>, 10 BaCl<sub>2</sub>, 10 HEPES, 10 glucose, 10 sucrose, 1 MgCl<sub>2</sub>, 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<sub>50</sub> values for nifedipine block were determined by fitting the fraction of current blocked at each drug concentration to the equation, Fraction Blocked =  $a/(1+([nifedipine]/IC_{50})^b)$  where a = maximum fraction blocked, b = slope. logEC<sub>50</sub> 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<sub>50</sub>), 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<sub>50</sub>), 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 logIC<sub>50</sub> and logEC<sub>50</sub> values ± S.E. of the fit shown in Table 1 are based on the fit of all of the data for a given channel construct. V<sub>1/2</sub> activation values were determined by plotting normalized tail current amplitudes vs. the corresponding 100 ms depolarizing voltage steps from -50 mV to +60 mV, in 10 mV increments, from a holding potential of -80 mV. The data were fit to the equation,  $I = 1/(1+\exp((V_{1/2}-V)/k))$  where k is a slope factor. The steady-state inactivation protocol used 10 sec conditioning pulses from -80 to +20 mV in 10 mV increments from a holding potential of -90 mV, followed by a 100 msec test pulse to +10 mV. V<sub>1/2</sub> inactivation was determined by plotting the normalized test pulse

amplitude vs. the conditioning pulse potential, and fitting the data to the equation  $I = 1/(1 + \exp(-(V - V_{1/2})/k))$  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<sub>v</sub>1.2 and Ca<sub>v</sub>1.3 based on the structure of Ca<sub>v</sub>1.1*- Homology models of Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3 were generated using SWISS-MODEL (Benkert et al., 2011; Bertoni et al., 2017; Bienert et al., 2017; Guex et al., 2009; Waterhouse et al., 2018). The structure of Ca<sub>v</sub>1.1 (PDB-code: 5gjjw) was used as template for modeling (Wu et al., 2016). Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3 share sequence identities of 72% and 71% with Ca<sub>v</sub>1.1, respectively.

*Data Analysis and Statistics*- Data were analyzed using Clampfit 10.6 (Axon Instruments) and SigmaPlot 11 (Systat Software, San Jose, CA).  $\log IC_{50}$  and  $\log EC_{50}$  values were determined using GraphPad Prism 7.04 (GraphPad Software, San Diego, CA). Comparisons of two means were made with Student's unpaired t-test. Comparisons of three or more means were made using one-way ANOVA.  $P < 0.05$  was considered significant. Data shown are means  $\pm$  S.E. Lines are fits of the equations indicated for each type of experiment to the data.

## Results

***Characterization of Ba<sup>2+</sup> current conducted by Ca<sub>v</sub>1.2 or Ca<sub>v</sub>1.3 co-expressed with the  $\beta_3$  and  $\alpha_2\delta_1$  subunits in tSA-201 cells***- We assessed the biophysical and pharmacological properties of Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3 in our expression system. As expected, Ca<sub>v</sub>1.3 activated at more negative voltages than Ca<sub>v</sub>1.2, ( $P < 0.001$ ) (Figure 1A; Table 1), and Ca<sub>v</sub>1.2 inactivated at slightly more negative voltages than Ca<sub>v</sub>1.3 ( $P < 0.01$ ) (Figure 1B; Table 1). We next examined the potency of nifedipine block of both channel types. We chose nifedipine because it's the most compact of the dihydropyridine Ca<sup>2+</sup> channel antagonists



(Supplemental Fig. 1), and our preliminary screen of several structurally distinct dihydropyridines revealed a substantial difference in nifedipine potency in blocking  $\text{Ca}_v1.2$  compared to  $\text{Ca}_v1.3$  (Supplemental Fig. 1). Channels were activated with 100 msec steps to +10 mV at a frequency of 0.033 Hz from a holding potential of -80 mV. After a baseline current was established, increasing concentrations of nifedipine were applied via a perfusion capillary in the bath solution. Figure 1C shows sample traces and the compiled dose response curves for both channel subtypes. As expected,  $\text{Ca}_v1.2$  was blocked more potently by nifedipine than  $\text{Ca}_v1.3$ , with  $\text{IC}_{50}$  values of  $22 \pm 2$  nM and  $289 \pm 30$  nM, respectively ( $P < 0.001$ ). The truncated splice variant  $\text{Ca}_v1.3_{42a}$  (Xu and Lipscombe, 2001), is reported to be less sensitive to nifedipine than the full-length  $\text{Ca}_v1.3_{42}$  variant (Huang et al., 2013). Therefore, we examined the dose-dependence of nifedipine block of  $\text{Ca}_v1.3_{42a}$ , and determined the  $\text{IC}_{50}$  for nifedipine to be  $436 \pm 24$  nM, greater than that of  $\text{Ca}_v1.3_{42}$  ( $P < 0.01$ ) (Figure 1C). We chose to use the full-length  $\text{Ca}_v1.3_{42}$  variant in the subsequent experiments, since it is structurally more similar to the  $\text{Ca}_v1.2$  variant used in this study.

***The III S5 transmembrane domain plays a key role in nifedipine block of  $\text{Ca}_v1.3$***  Studies in  $\text{Ca}_v1.2$  have established transmembrane domain III S5 as a key component of the DHP binding pocket (Mitterdorfer et al., 1996). Specifically, mutations of T1039 and Q1043 (underlined in Figure 2A) to the corresponding residues in DHP-insensitive voltage-gated  $\text{Ca}^{2+}$  channels results in a  $\text{Ca}_v1.2$  mutant channel (termed  $\text{Ca}_v1.2/\text{DHPi}$ ) that is markedly less sensitive to DHPs, but normally sensitive to diltiazem (Hockerman et al., 2000; Lin et al., 2011). We made the corresponding  $\text{Ca}_v1.3/\text{DHPi}$  mutant, and as expected, it was substantially less sensitive to nifedipine than  $\text{Ca}_v1.3$  (Figure 2B). In fact, we were unable to determine the maximum percent of  $\text{Ca}_v1.3/\text{DHPi}$  current blocked because the nifedipine concentrations at the high end of the range ( $>200$   $\mu\text{M}$ ) were at the limit of aqueous solubility (Ran et al., 2002). Assuming maximal inhibition of 90% of current, we estimated the  $\text{IC}_{50}$  of nifedipine block of  $\text{Ca}_v1.3/\text{DHPi}$  to be  $\sim 93$   $\mu\text{M}$ , more than 300x that for  $\text{Ca}_v1.3$ . As with the corresponding mutation in  $\text{Ca}_v1.2$  (Hockerman, et al., 2000), the

sensitivity of Ca<sub>v</sub>1.3/DHPi to block by the BTZ diltiazem was not reduced compared to Ca<sub>v</sub>1.3

(Supplemental Fig. 2).

Given that transmembrane domain IIS5 clearly contributes to the DHP binding pocket in Ca<sub>v</sub>1.3, we next examined the single amino acid in this domain that is not conserved between Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3, M1030 (Figure 2A). The corresponding position in Ca<sub>v</sub>1.2 (1036) is occupied by a V residue, so we constructed the mutant channel Ca<sub>v</sub>1.3/MV, to determine if this conservative change could contribute to the difference in nifedipine potency between Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3. The V<sub>1/2</sub> inactivation of Ca<sub>v</sub>1.3/MV was not different from that of Ca<sub>v</sub>1.3; however, the V<sub>1/2</sub> activation of Ca<sub>v</sub>1.3/MV (-26 ± 1.1) (Table 1) was more positive than that for Ca<sub>v</sub>1.3 (*P* < 0.05). The M1030V mutation increased the potency of nifedipine block of Ca<sub>v</sub>1.3, reducing the IC<sub>50</sub> from 289 nM to 89 ± 7 nM (*P* < 0.001) (Figure 2C). Given that this relatively conservative change in structure shifted the potency of nifedipine block of Ca<sub>v</sub>1.3 toward those of Ca<sub>v</sub>1.2, we asked if the reciprocal change in Ca<sub>v</sub>1.2 (Ca<sub>v</sub>1.2/VM) would shift the potency of nifedipine block toward that of Ca<sub>v</sub>1.3. Indeed, we found that the V1036M mutation increased the IC<sub>50</sub> of nifedipine for block of current compared to Ca<sub>v</sub>1.2 (39 ± 6 nM) (*P* < 0.05) (Figure 2D). In addition, the V<sub>1/2</sub> activation of Ca<sub>v</sub>1.2/VM was -24 ± 1 mV, more negative than that for Ca<sub>v</sub>1.2 (*P* < 0.01) (Figure 2E; Table 1). Thus, this single, conservative difference between Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3 in transmembrane segment IIS5 contributes to differences in both voltage-dependence of activation and nifedipine potency between these two channels.

***The extracellular domain IIS5-3P contributes to the difference in nifedipine potency between Ca<sub>v</sub>1.2***

***and Ca<sub>v</sub>1.3-*** Given that the small difference in amino acid sequence between Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3 in IIS5 only partially accounts for the difference in nifedipine potency, we next examined the role of the extracellular domain just downstream of IIS5, the IIS5-3P loop. This region is an area of relatively high amino acid sequence divergence between Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3 (Figure 3A), and some determinants of DHP potency/affinity have been identified in this region. Therefore, we created a chimeric channel, Ca<sub>v</sub>1.3+, which incorporates the Ca<sub>v</sub>1.2 IIS5-3P loop into the Ca<sub>v</sub>1.3 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 Ca<sub>v</sub>1.3+ compared to Ca<sub>v</sub>1.3 (see Table 1). However, the IC<sub>50</sub> for nifedipine block of Ca<sub>v</sub>1.3+ (101 ± 4 nM) was reduced compared to that for Ca<sub>v</sub>1.3 (*P* < 0.001) (Figure 3B).

We next asked if a particular region of the IIS5-3P loop could account for the increase in nifedipine potency in block of Ca<sub>v</sub>1.3+ versus Ca<sub>v</sub>1.3. The IIS5-3P loop extends from the end of IIS5 to the conserved E residue in the domain III selectivity filter (Figure 3A). The region just upstream of the conserved selectivity filter E residue of homologous domain IIII (Yang et al., 1993)(1118 in Ca<sub>v</sub>1.2, 1112 in Ca<sub>v</sub>1.3; Figure 3A) is known to be involved in DHP modulation of Ca<sub>v</sub>1.2 (Yamaguchi et al., 2000; Yamaguchi et al., 2003), but is highly conserved between Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3. Mutation of the nearest non-conserved residue upstream of E1112 in Ca<sub>v</sub>1.3 (S1100) resulted in a channel (Ca<sub>v</sub>1.3/SA) with V<sub>1/2</sub> activation not different from Ca<sub>v</sub>1.3, but with markedly left-shifted V<sub>1/2</sub> inactivation (see Table 1). The IC<sub>50</sub> for nifedipine block of Ca<sub>v</sub>1.3/SA was 99 ± 24 nM, indistinguishable from that for Ca<sub>v</sub>1.3+ (Figure 3B). The IIS5-3P loop of both Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3 contain two P residues, one of which is conserved (1081/1087), and another that differs significantly in position relative to the conserved P residue (P1063 in Ca<sub>v</sub>1.3 and P1091 in Ca<sub>v</sub>1.2) (See Figure 3A). We reasoned that this difference in P configuration could affect the conformation of the conserved, distal portion of the IIS5-3P loop, and thus DHP affinity. Therefore, we created Ca<sub>v</sub>1.3/PEEP, with P residues at position 1081 and 1085, but a P to E switch at position 1063, mimicking the P configuration of the Ca<sub>v</sub>1.2 IIS5-3P loop. The voltage-dependence of inactivation was not different from Ca<sub>v</sub>1.3, and the voltage-dependence of activation was ~3 mV more negative than Ca<sub>v</sub>1.3 (see Table 1). The IC<sub>50</sub> for nifedipine block of Ca<sub>v</sub>1.3/PEEP was 188 ± 28 nM, not statistically significantly lower than the IC<sub>50</sub> for block of Ca<sub>v</sub>1.3 (Figure 3C, Table 1). However, the Hill slope for the dose response curve for nifedipine block of Ca<sub>v</sub>1.3PEEP (0.43 ± 0.02) was shallower than Ca<sub>v</sub>1.3 (*P* < 0.001). We next turned our attention to a region of the IIS5-3P loop proximal to IIS5 that contains a cluster of three negatively charged residues in Ca<sub>v</sub>1.2 (D1063, E1069, E1071), reported to affect DHP binding affinity

(Wang et al., 2007). Only two of these negative charges are conserved in Ca<sub>v</sub>1.3 (D1057&E1065); moreover, the amino acid sequence surrounding these residues is highly divergent between Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3 (see Figure 3A). Therefore, we created the mutant Ca<sub>v</sub>1.3/N6 with the Ca<sub>v</sub>1.2 sequence from amino acid 1064-1070 (SSKQTEA) inserted into the corresponding position (1058-1064) in Ca<sub>v</sub>1.3. We found that expression of Ca<sub>v</sub>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 Ca<sub>v</sub>1.3/N6, which restored inward barium current. The voltage-dependence of activation of Ca<sub>v</sub>1.3/N6 under 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<sub>50</sub> for nifedipine block of Ca<sub>v</sub>1.3/N6 ( $116 \pm 53$  nM) was slightly ( $P < 0.05$ ) lower than that for Ca<sub>v</sub>1.3, but the Hill slope of the dose response curve ( $0.52 \pm 0.1$ ) was also less than Ca<sub>v</sub>1.3 ( $P < 0.05$ ) (Figure 3D).

Given that the decreases in nifedipine IC<sub>50</sub> for both Ca<sub>v</sub>1.3/MV and Ca<sub>v</sub>1.3+ were relatively modest, we asked if combining these mutations would further increase the potency of nifedipine block. The V<sub>1/2</sub> activation of the resulting mutant channel, Ca<sub>v</sub>1.3+V, was not different from Ca<sub>v</sub>1.3, but the V<sub>1/2</sub> inactivation was shifted by -6 mV (see Table 1). However, the IC<sub>50</sub> for nifedipine block of Ca<sub>v</sub>1.3+V was reduced to  $42 \pm 5$  nM (Figure 3E), compared to  $289 \pm 30$  nM for Ca<sub>v</sub>1.3 ( $P < 0.001$ ), but was still greater than the IC<sub>50</sub> of nifedipine for Ca<sub>v</sub>1.2 ( $P < 0.05$ ). Thus, amino acid differences in the IIS5-3P loop, along with the single amino acid divergence in IIS5, account for the vast majority of the difference in potency of nifedipine block of Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3.

We next asked if the small remaining gap in nifedipine potency between Ca<sub>v</sub>1.3+V and Ca<sub>v</sub>1.2 could be closed. Besides IIS5 and the IIS5-3P loop, transmembrane domain IIS6 also contributes to the DHP binding pocket in Ca<sub>v</sub>1.2 (Hockerman et al., 1997b). The only amino acid residue in IIS6 not conserved between Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3 is an I/V divergence at position 1156/1150; moreover, mutation of I1156 in Ca<sub>v</sub>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 IIS6 of either Ca<sub>v</sub>1.3+V or Ca<sub>v</sub>1.3/MV resulted in channels that yielded little to no current upon expression in tSA201 cells.

***Differences in the IIS5-3P loop are responsible for the difference in potency of FPL in Ca<sub>v</sub>1.2 and***

***Ca<sub>v</sub>1.3*** The non-dihydropyridine compound FPL 64176 (FPL) (Ginap et al., 1993) is a well-characterized potentiator of Ca<sub>v</sub>1.2 current (Liu et al., 2003). Reconstruction of the DHP binding site in the P/Q-type channel Ca<sub>v</sub>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<sub>v</sub>1.3. Therefore, we compared the potency of FPL potentiation of current in Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3. The experiments with Ca<sub>v</sub>1.3 utilized balanced NMDG solutions because we found that application of FPL frequently induced outward current when the extracellular solution contained no NMDG (Supplemental Figure 3A), suggesting that FPL binding substantially affects the permeability of Ca<sub>v</sub>1.3 to NMDG. We found that the EC<sub>50</sub> for potentiation of current amplitude in Ca<sub>v</sub>1.2 by FPL was 102 ± 40 nM (Figure 4A&D). In contrast, the EC<sub>50</sub> for potentiation of Ca<sub>v</sub>1.3 current amplitude by FPL was 854 ± 236 nM (*P* < 0.05) (Figure 4B&D). Thus, as with nifedipine, Ca<sub>v</sub>1.3 is less sensitive to FPL than Ca<sub>v</sub>1.2.

We next asked if some of the same differences between Ca<sub>v</sub>1.2 and Ca<sub>v</sub>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<sub>v</sub>1.3+V, since this mutant had nearly the same sensitivity to nifedipine as Ca<sub>v</sub>1.2. We were able to perform these experiments in the standard solution set, since FPL did not induce outward current in Ca<sub>v</sub>1.3+V. The EC<sub>50</sub> for potentiation of current amplitude by FPL in Ca<sub>v</sub>1.3+V was 99 ± 5 nM (Figure 4 C&D), indistinguishable from the EC<sub>50</sub> of FPL for potentiation of Ca<sub>v</sub>1.2. We measured the EC<sub>50</sub> for FPL potentiation of Ca<sub>v</sub>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 EC<sub>50</sub> of FPL for Ca<sub>v</sub>1.3/MV was 737 ± 20 nM, not different from the EC<sub>50</sub> for

Ca<sub>v</sub>1.3 (Figure 4D). Taken together, these results suggest that the molecular determinants of the difference in potency of FPL lie within the IIS5-3P loop. Ca<sub>v</sub>1.3+ exhibited outward current in the presence of FPL, similar to Ca<sub>v</sub>1.3 (Supplemental Figure 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 Figure 3B). We were able to measure the potency of FPL potentiation of the IIS5-3P loop mutants Ca<sub>v</sub>1.3/PEEP, Ca<sub>v</sub>1.3/N6, and Ca<sub>v</sub>1.3/SA, and found that none of these mutants displayed increased sensitivity to potentiation of current by FPL compared to Ca<sub>v</sub>1.3 (Table 1). Thus, we have identified two regions of amino acid divergence between Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3 within the IIS5-3P loop, Ca<sub>v</sub>1.2 1106/ Ca<sub>v</sub>1.3 1100 and Ca<sub>v</sub>1.2 1064-1070/Ca<sub>v</sub>1.3 1058-64, that appear to confer differences in sensitivity to nifedipine block, but not 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<sub>v</sub>1.2 and Ca<sub>v</sub>1.3. Figure 5 shows the effect of 10 μM FPL on tail current kinetics, a measure of the rate of deactivation. Ca<sub>v</sub>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 (Figure 5A; Table 2). In contrast, deactivation in Ca<sub>v</sub>1.2 in the absence of FPL follows two  $\tau$ s. However, a single slow  $\tau$  is principally observed in the presence of FPL which is greater than both  $\tau$ s in the absence of FPL (Figure 5B, Table 2). Given the differences in the kinetics of deactivation in Ca<sub>v</sub>1.3 vs Ca<sub>v</sub>1.2, we compared the FPL-induced slowing of deactivation in these channels by measuring the fraction of the tail current remaining 10 msec after reaching peak (R10). The R10 for both Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3 in the absence of FPL was negligible. Figure 5C shows that the R10 of Ca<sub>v</sub>1.2 in the presence of 10 μM FPL ( $0.67 \pm 0.09$ ) was greater than that of Ca<sub>v</sub>1.3 ( $0.15 \pm 0.02$ ) ( $P < 0.001$ ), indicating a greater slowing of deactivation by FPL in Ca<sub>v</sub>1.2. We also found that FPL shifted the  $V_{1/2}$  activation of Ca<sub>v</sub>1.2 by -26 mV, but only -10 mV in Ca<sub>v</sub>1.3 (Table 2). Thus, FPL is not only more potent in stimulating current amplitude in Ca<sub>v</sub>1.2 compared to Ca<sub>v</sub>1.3, but also has stronger effects on deactivation kinetics and the voltage-dependence of activation in Ca<sub>v</sub>1.2 at a maximally

effective concentration (10  $\mu$ M).

We next asked if the Ca<sub>v</sub>1.3/ DHPi channel was less sensitive to FPL than Ca<sub>v</sub>1.3. Deactivation of Ca<sub>v</sub>1.3/DHPi followed a single  $\tau$  that was not different from that of Ca<sub>v</sub>1.3 (Table 2), but was not altered by 10  $\mu$ M FPL (Figure 5D & E). Not surprisingly, no significant increase in current was observed upon application of 10  $\mu$ M FPL to Ca<sub>v</sub>1.3DHPi. Interestingly, 10  $\mu$ M FPL did shift the  $V_{1/2}$  activation of Ca<sub>v</sub>1.3DHPi by -9 mV (Table 2). Since IIIIS5 is clearly crucial for the action of FPL, we examined the kinetics of deactivation in Ca<sub>v</sub>1.3/MV (Figure 5F). In the absence of FPL, Ca<sub>v</sub>1.3/MV deactivation followed a single  $\tau$  ( $0.71 \pm 0.04$  msec) that was slightly, but statistically significantly greater than Ca<sub>v</sub>1.3 ( $0.41 \pm 0.07$  msec) ( $P < 0.01$ ) (Figure 5G). In the presence of 10  $\mu$ M FPL, the R10 was greater in Ca<sub>v</sub>1.3/MV ( $0.52 \pm 0.15$  msec)( $P < 0.05$ ) compared to Ca<sub>v</sub>1.3 (Figure 5H). However, deactivation of Ca<sub>v</sub>1.2/VM was not different from that of Ca<sub>v</sub>1.2 either in the absence or presence of FPL (Table 2). Thus, the M to V switch at position 1030 of Ca<sub>v</sub>1.3 does not affect FPL potency, but does affect both deactivation and slowing of deactivation by FPL.

## Discussion

The voltage-dependence and sensitivity to nimodipine (a DHP antagonist) of the Ca<sub>v</sub>1.3 cDNA used in this study (Ca<sub>v</sub>1.3<sub>42</sub>; Xu and Lipscomb, 2001) was previously characterized. The  $V_{1/2}$  activation reported here is indistinguishable from that initial characterization. Further, Xu and Lipscombe reported an ~20 fold higher IC<sub>50</sub> for nimodipine block of Ca<sub>v</sub>1.3 compared to Ca<sub>v</sub>1.2. For our comparison of DHP antagonist potency, we chose nifedipine 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 IC<sub>50</sub> for nifedipine block of Ca<sub>v</sub>1.3 compared to Ca<sub>v</sub>1.2 is in line with the decreased potency of nimodipine in block of Ca<sub>v</sub>1.3 compared to Ca<sub>v</sub>1.2 reported by Xu and Lipscombe. Though they did not report an EC<sub>50</sub> for agonist potentiation of Ca<sub>v</sub>1.3, Xu and Lipscombe did

report a modest shift in  $V_{1/2}$  activation of  $Ca_v1.3$  by 1  $\mu$ M of the DHP agonist Bay K 8644 ( $\sim$ -7 mV), similar to the modest leftward shift in  $V_{1/2}$  activation we observed in  $Ca_v1.3$  in the presence of 10  $\mu$ M FPL. Thus, our data show that our expression system recapitulates the primary differences between  $Ca_v1.2$  and  $Ca_v1.3$ , most notably, the left-shifted activation and lower sensitivity of current to block by DHP antagonists of  $Ca_v1.3$  compared to  $Ca_v1.2$ .

Another study examined both the binding affinity and block potency of the DHP antagonist PN200-110 (isradipine) for a  $Ca_v1.3$  clone from human pancreas ( $Ca_v1.3_{8A}$ ) (Koschak et al., 2001). Interestingly, the  $K_D$  for [ $^3$ H]PN200-110 binding was not significantly different between  $Ca_v1.3_{8A}$  and  $Ca_v1.2$  cloned from rabbit cardiac muscle (Tanabe et al., 1987). However, the  $IC_{50}$  for block of current by PN200-110 was reported to be 8.5 fold higher for  $Ca_v1.3_{8A}$  than  $Ca_v1.2$ , in excellent agreement with the difference in nifedipine potency in blocking  $Ca_v1.3$  and  $Ca_v1.2$  in this study.  $K_D$  values for binding of DHPs to L-type channels in isolated membranes are invariably lower than  $IC_{50}$  values for current block. For example, the  $K_D$  for binding of [ $^3$ H]-PN200-110 binding to the  $Ca_v1.2$  clone used in this study is 55 pM, while the  $IC_{50}$  for PN200-110 block is 7 nM (Peterson et al., 1997). Binding isotherms in both studies clearly indicated a single [ $^3$ H]-PN200-110 binding site, which likely reflects the open, inactivated state of the channel at 0 mV. Thus, it is likely that the Hill slopes different from 1 that we observed for nifedipine block of  $Ca_v1.3$  and some of the mutant channels used in this study reflect the presence of distinct voltage-dependent channel conformations which regulate DHP affinity.

Though the DHP binding pockets of  $Ca_v1.2$  and  $Ca_v1.3$  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  $Ca_v1.2$  and  $Ca_v1.3$ . The IIIS5 helix is clearly a critical component of the  $Ca_v1.3$  DHP binding pocket, as mutation of T1033 and Q1037 in  $Ca_v1.3$ /DHPi results in a marked loss of nifedipine potency. The side chains of M1030/V1036 in  $Ca_v1.3$  and  $Ca_v1.2$  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 Ca<sub>v</sub>1.3-specific residue at this position in to Ca<sub>v</sub>1.2 (V1036M) shifts nifedipine potency toward that of Ca<sub>v</sub>1.3, and vice versa. Interestingly, the swap of channel subtype-specific residues in this position also results in small reciprocal shifts in V<sub>1/2</sub> activation (Table 1). However, only the Ca<sub>v</sub>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<sub>v</sub>1.3 affects voltage-dependence of activation and the rate of tail current decay, suggests that position 1030 in IIS5 (outer pore helix) may interact with IIS6 (inner pore helix) in a manner that regulates channel gating. Previously published models of DHP binding in Ca<sub>v</sub>1.2 suggest that amino acid residues directly interacting with DHP drugs are conserved between Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3 (Tikhonov DB and Zhorov, 2009) (Cosconati et al., 2007). To understand how subtle differences in amino acid sequence might account for a significant difference in nifedipine potency, we constructed homology models of Ca<sub>v</sub>1.3 and Ca<sub>v</sub>1.2 (Figure 6) based on the recently published high-resolution Cryo-EM structure of Ca<sub>v</sub>1.1 (Wu et al., 2016). The models suggest that the increase in side chain bulk between Ca<sub>v</sub>1.3 and Ca<sub>v</sub>1.2 at position 1030/1036 (M vs V) could potentially decrease accessibility of nifedipine to the critical Q1037 and F1106 residues (Figure 6A). In addition, the model predicts that S1100 in Ca<sub>v</sub>1.3 can form a hydrogen bond with N1094, an interaction that could potentially constrain the movement of the 3P helix during nifedipine binding (Figure 6B). The corresponding positions in Ca<sub>v</sub>1.2 are occupied by an alanine residue (1106) and a glutamate (1100), precluding such an interaction (Figure 6C). Interestingly, S1100 of Ca<sub>v</sub>1.3 is conserved in the corresponding position of Ca<sub>v</sub>1.1 (S1002) and the position corresponding to N1094 of Ca<sub>v</sub>1.3 is a histidine in Ca<sub>v</sub>1.1 (H996). These residues, with the assistance of D998, may form a hydrogen bond in Ca<sub>v</sub>1.1 (Figure 6D), which may contribute to the lower binding affinity of Ca<sub>v</sub>1.1 for [<sup>3</sup>H]-PN200-110 (270 pM)(Peterson et al., 1996) compared to Ca<sub>v</sub>1.2 (55 pM) (Peterson et al., 1997). Thus, our model suggests that the effect of the Ca<sub>v</sub>1.3S/A mutation on nifedipine potency is indirect, and that the displacement of the 3P helix may be required for high potency block of Ca<sub>v</sub>1.2 by DHP drugs.

Our studies of FPL potentiation of  $Ca_v1.2$ ,  $Ca_v1.3$ , and the various mutant channels also yielded some novel results. First, Figure 4 clearly shows that FPL is much more potent in potentiating current conducted by  $Ca_v1.2$  compared to  $Ca_v1.3$ . This difference can be ascribed completely to amino acid differences in the IIS5-3P loop between these two channels. Nevertheless, the conserved T and Q residues in IIS5 are clearly important for FPL action on  $Ca_v1.3$  even though the nearby M1030V mutation did not increase the potency of FPL action in isolation. However, the inclusion of V1030 in  $Ca_v1.3+V$  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  $Ca_v1.3/DHPi$  was not different from that of  $Ca_v1.3$ , suggesting distinct sites of action on  $Ca_v1.3$  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  $Ca_v1.2$  and  $Ca_v1.3$  beyond the IIS5-3P loop, as none of the mutations within this domain that increased nifedipine potency improved FPL potency at  $Ca_v1.3$ . It's possible that these determinants may be among the sixteen other amino acid differences between  $Ca_v1.2$  and  $Ca_v1.3$  within this domain that we did not examine.

In our studies of the  $Ca_v1.3/N6$  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  $Ca_v1.2$  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., 1995). Indeed, we found that, by equalizing the NMDG concentration in the intra- and extracellular solutions, the outward current observed in the  $Ca_v1.3/N6$  mutant was abolished, and we were able to complete the biophysical and pharmacological measurements reported in Table 1. Similarly, we found that  $Ca_v1.3$  and the  $Ca_v1.3+$  mutant tended to undergo current reversal upon FPL application (Supplemental Figure 3A) that was abolished in  $Ca_v1.3$  by equalizing the NMDG concentrations.

However, even this maneuver left unstable current when FPL was applied to Ca<sub>v</sub>1.3+, and we were unable to determine an EC<sub>50</sub> for FPL stimulation of this mutant (Supplemental Figure 3B). FPL was previously reported to alter the permeability of Ca<sub>v</sub>1.2 (Fan et al., 2001), such that Cd<sup>2+</sup> became a permeant ion, rather than a pore blocker, in the absence of Ca<sup>2+</sup>. Thus, our observation that FPL can induce NMDG permeability in Ca<sub>v</sub>1.3 is consistent with the notion that FPL binding may induce conformational changes in the IIIS5-3P loop that affect the ion selectivity of Ca<sub>v</sub>1.3. Interestingly, neither the Ca<sub>v</sub>1.3+V nor the Ca<sub>v</sub>1.3M/V mutant conducted outward current in the presence of FPL in the standard solution set, suggesting that the M1030 residue may play a role in the observed permeability changes in Ca<sub>v</sub>1.3.

In summary, this study demonstrates that the reduced sensitivity of Ca<sub>v</sub>1.3 to both nifedipine and FPL compared to Ca<sub>v</sub>1.2 can be largely attributed to amino acid differences within the previously defined DHP binding pocket. In the case of nifedipine, this difference can be attributed to the M/V divergence in transmembrane domain IIIS5, and an S/A divergence in the IIIS5-3P loop. Our homology models suggest that divergence in IIIS5 results in distinct steric effects on drug binding, while the divergence in the IIIS5-3P loop may regulate displacement of the 3P helix upon ligand binding.

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## **Authorship Contributions**

*Participated in research design:* Wang and Hockerman

*Conducted experiments:* Wang, Tang, Harvey, and Hockerman

*Contributed new reagents or analytical tools:* Li, Rantz, Salyer, and Lill

*Performed data analysis:* Wang, Tang, Harvey and Hockerman

*Wrote or contributed to the writing of the manuscript:* Wang and Hockerman

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## Footnotes

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## Figure Legends

### Figure 1. Characterization of Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3 biophysical properties and nifedipine block- **A)**

Determination of the voltage-dependence of activation in Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3. V<sub>1/2</sub> activation values were -20 ± 0.5 mV for Ca<sub>v</sub>1.2 (N = 6) and -30 ± 1.5 mV for Ca<sub>v</sub>1.3 (N = 9) (*P* < 0.001). **B)** Determination of the voltage-dependence of inactivation in Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3. V<sub>1/2</sub> inactivation values were -41 ± 0.6 mV for Ca<sub>v</sub>1.2 (N = 6) and -36 ± 1.3 mV (N = 5) for Ca<sub>v</sub>1.3 (*P* < 0.01). **C)** Determination of potency of nifedipine block of Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3. The IC<sub>50</sub> values of nifedipine block were 22 ± 2 nM (N = 3-12) for Ca<sub>v</sub>1.2 and 289 ± 30 nM (N = 7) for Ca<sub>v</sub>1.3 (*P* < 0.001). Example traces from experiments are shown at right. The IC<sub>50</sub> for nifedipine block of Ca<sub>v</sub>1.3<sub>42a</sub>, a truncated splice variant, was 436 ± 24 nM (N = 5-6), statistically significantly greater than that of Ca<sub>v</sub>1.3 (*P* < 0.01).

### Figure 2. Contribution of transmembrane domain IIIS5 to nifedipine block of Ca<sub>v</sub>1.3- **A)** Amino

acid sequence alignment of the IIIS5 transmembrane domains in Ca<sub>v</sub>1.3, Ca<sub>v</sub>1.2, and the mutant Ca<sub>v</sub>1.3/DHPI. The only difference is the M to V switch at position 1030/1036 (in red). The underlined residues were mutated to create Ca<sub>v</sub>1.3/DHPI, and are critical for dihydropyridine block of Ca<sub>v</sub>1.2. **B)** Nifedipine dose-response curve for block of Ca<sub>v</sub>1.3/DHPI. The IC<sub>50</sub> of nifedipine for Ca<sub>v</sub>1.3/DHPI was estimated at ~93 μM. **C)** Dose-response curve for nifedipine block of Ca<sub>v</sub>1.3/MV. The IC<sub>50</sub> value was 89 ± 7 nM (N = 5-7), less than the IC<sub>50</sub> of nifedipine block of Ca<sub>v</sub>1.3 (*P* < 0.001). **D)** Dose-response curve for nifedipine block of Ca<sub>v</sub>1.2/VM. The IC<sub>50</sub> value was 39 ± 5nM (N = 4-6), greater than the IC<sub>50</sub> for block of Ca<sub>v</sub>1.2 (*P* < 0.05). **E)** Voltage-dependent activation of Ca<sub>v</sub>1.2/VM. The V<sub>1/2</sub> activation for Ca<sub>v</sub>1.2/VM was -24 ± 1 mV (N = 8), more negative than that for Ca<sub>v</sub>1.2 (*P* < 0.05).

### Figure 3. Contribution of the IIIS5-3P loop to nifedipine block of Ca<sub>v</sub>1.3- **A)** Amino acid sequence

alignment of the extracellular IIIS5-3P loops of Ca<sub>v</sub>1.2 (aa 1058-1118) and Ca<sub>v</sub>1.3 (aa 1052-1112).

Twenty-four of the sixty amino acids in this segment are not conserved. Identities are indicated with a dash. Asterisks indicate amino acid residues previously reported to influence dihydropyridine modulation of  $\text{Ca}_v1.2$ . The  $\text{Ca}_v1.3+$  mutant incorporated all of the  $\text{Ca}_v1.2$ -specific amino acids in this segment into  $\text{Ca}_v1.3$ . The  $\text{Ca}_v1.3+V$  mutant is  $\text{Ca}_v1.3+$  combined with the substitution of V for M at position 1030. The  $\text{Ca}_v1.3/SA$  mutant incorporated only the S to A substitution at position 1100. The  $\text{Ca}_v1.3/PEEP$  mutation incorporated the substitution of P for E and E for P at positions 1063 and 1085, respectively. The  $\text{Ca}_v1.3/N6$  mutation incorporated the six  $\text{Ca}_v1.2$ -specific amino acid residues from position 1064 to 1070 (boxed residues) into  $\text{Ca}_v1.3$ . **B)** Dose-response curve for block of  $\text{Ca}_v1.3+$  and  $\text{Ca}_v1.3/SA$  by nifedipine.  $\text{Ca}_v1.3+$  (black circles) and  $\text{Ca}_v1.3/SA$  (gray circles) were both more sensitive to block by nifedipine than  $\text{Ca}_v1.3$  ( $P < 0.001$ ,  $P < 0.01$ , respectively).  $\text{IC}_{50}$  for nifedipine block of:  $\text{Ca}_v1.3+ = 101 \pm 4$  nM ( $N = 6-8$ );  $\text{Ca}_v1.3/SA = 99 \pm 24$  nM ( $N = 4-5$ ). **C)** Dose response curve for block of  $\text{Ca}_v1.3/PEEP$  by nifedipine. The  $\text{IC}_{50}$  for nifedipine block of  $\text{Ca}_v1.3/PEEP$  was  $188 \pm 28$  nM ( $N = 3-7$ ), not different from that of  $\text{Ca}_v1.3$ ; however, the Hill slope ( $0.43 \pm 0.02$ ), was shallower than that for  $\text{Ca}_v1.3$  ( $0.78 \pm 0.04$ ) ( $P < 0.001$ ). **D)** Dose response curve for block of  $\text{Ca}_v1.3/N6$  by nifedipine. The  $\text{IC}_{50}$  for nifedipine block of  $\text{Ca}_v1.3/N6$  was  $116 \pm 53$  nM ( $N = 5-9$ ), lower than that of  $\text{Ca}_v1.3$  ( $P < 0.05$ ). The Hill slope was ( $0.52 \pm 0.10$ ), shallower than that for  $\text{Ca}_v1.3$  ( $0.78 \pm 0.04$ ) ( $P < 0.05$ ). **E)** Dose response curve for nifedipine block of  $\text{Ca}_v1.3+V$  compared to those for  $\text{Ca}_v1.2$  and  $\text{Ca}_v1.3$ . The  $\text{IC}_{50}$  for nifedipine block of  $\text{Ca}_v1.3+V$  was  $42 \pm 5$  nM ( $N = 4-10$ ), lower than that for  $\text{Ca}_v1.3$  ( $P < 0.001$ ).

**Figure 4. Potency of FPL 64176 potentiation of  $\text{Ca}_v1.2$ ,  $\text{Ca}_v1.3$ , and mutant channels- A-C)**

Example traces showing FPL potentiation of  $\text{Ca}_v1.2$ ,  $\text{Ca}_v1.3$ , and  $\text{Ca}_v1.3+V$ , respectively. Note the marked slowing of the tail current in  $\text{Ca}_v1.2$  that is absent in  $\text{Ca}_v1.3$ . **D)** Dose response curves for FPL 64176 potentiation of  $\text{Ca}_v1.2$ ,  $\text{Ca}_v1.3$ , and mutant channels. The  $\text{EC}_{50}$  values for FPL potentiation of current for  $\text{Ca}_v1.2$  and  $\text{Ca}_v1.3$  were  $103 \pm 40$  nM ( $N = 3-8$ ) and  $854 \pm 236$  nM ( $N = 3-7$ ), respectively ( $P < 0.05$ ). The

EC<sub>50</sub> for FPL potentiation of the mutant Ca<sub>v</sub>1.3+V (99 ± 5 nM) (N = 3-7) was not different from that of Ca<sub>v</sub>1.2, but was different from that of Ca<sub>v</sub>1.3 (*P* < 0.05). In contrast, the EC<sub>50</sub> for FPL potentiation of the mutant Ca<sub>v</sub>1.3/MV was 737 ± 20 nM (N = 5), not different from that of Ca<sub>v</sub>1.3. Data are shown as the mean fractional increase in current compared to 10 μM FPL 64176 ± SE.

**Figure 5. Kinetics of tail current decay in the presence and absence of FPL 64176 in Ca<sub>v</sub>1.2, Ca<sub>v</sub>1.3, and mutant channels-** **A)** Example 100 ms depolarization demonstrating tail current decay in Ca<sub>v</sub>1.3 in the presence or absence of 10 μM FPL 64176. **B)** Example 100 ms depolarization demonstrating tail current decay in Ca<sub>v</sub>1.2 in the presence or absence of 10 μM FPL 64176. **C)** The R10 value (fraction of tail current remaining 10 msec after peak) in the presence of FPL was greater in Ca<sub>v</sub>1.2 (0.67 ± 0.09, N = 6) compared to that of Ca<sub>v</sub>1.3 (0.15 ± 0.02) (N = 5) (\*\*\*, *P* < 0.001). **D)** Example 100 ms depolarization demonstrating tail current decay in Ca<sub>v</sub>1.3/DHPi in the presence or absence of 10 μM FPL 64176. **E)** The time constant for deactivation of Ca<sub>v</sub>1.3/DHPi (τ = 0.59 ± 0.11 msec) (N = 5) was not affected by the presence of 10 μM FPL (τ = 0.60 ± 0.04 msec) (N = 5). **F)** Example 100 ms depolarization demonstrating tail current decay in Ca<sub>v</sub>1.3/MV in the presence or absence of 10 μM FPL 64176. **G)** The time constant for deactivation of Ca<sub>v</sub>1.3/MV in the absence of FPL followed a single time constant (τ = 0.70 ± 0.13 msec) (N = 5) that was slower than that of Ca<sub>v</sub>1.3 (\*\*, *P* < 0.01). **H)** The R10 value for Ca<sub>v</sub>1.3/MV tail current in the presence of 10 μM FPL (0.51 ± 0.15) (N = 5) was greater than that of Ca<sub>v</sub>1.3 (\*, *P* < 0.05).

**Figure 6. Influence of Ca<sub>v</sub>1.3-specific amino acid residues on the DHP binding pocket-** Homology models of Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3 were created based on the high-resolution CryoEM structure of Ca<sub>v</sub>1.1. **A)** View of the DHP binding pocket of Ca<sub>v</sub>1.3 framed by the IIIS5 helix (bottom), IVS6 helix (top), and the 3P helix (right) with V1036 from Ca<sub>v</sub>1.2 superimposed on M1030. **B)** View of the backside of the 3P helix in

Ca<sub>v</sub>1.3 showing a potential H-bond between the Ca<sub>v</sub>1.3-specific residues S1100 and N1094. **C)** View of the backside of the 3P helix in Ca<sub>v</sub>1.2 with the positions of A1106 and D1100 indicated. **D)** View of the backside of the 3P helix in Ca<sub>v</sub>1.1 showing potential a H-bond between S1002 and H996 facilitated by D998.

Table 1. Pharmacology and Voltage-Dependence of Cav1.2, Cav1.3, and Mutant Channels

| Channel (+ $\beta_3$ & $\alpha_2\delta_1$ ) | Nifedipine Log IC <sub>50</sub> | Nifedipine Hill slope | Nifedipine Max (%) | FPL64176 Log EC <sub>50</sub> | V <sub>1/2</sub> inact. (mV) | V <sub>1/2</sub> act. (mV) | $\Delta$ V <sub>1/2</sub> act. FPL (mV) |
|---|---------------------------------|-----------------------|--------------------|-------------------------------|------------------------------|----------------------------|---|
| Ca <sub>v</sub> 1.2                         | -7.59 ± 0.03<br>N = 3-12        | 1.0 ± 0.1             | 90 ± 3             | -6.95 ± 0.16<br>N = 3-8       | -41 ± 0.6<br>N = 6           | -20 ± 0.5<br>N = 6         | -26 ± 0.7<br>N = 7                      |
| Ca <sub>v</sub> 1.3 (long)                  | -6.54 ± 0.04<br>N = 7           | 0.78 ± 0.05           | 88 ± 2             | -6.05 ± 0.08*<br>N = 3-7      | -36 ± 1.3<br>N = 5           | -30 ± 1.5<br>N = 9         | -10.2 ± 1.8<br>N = 9                    |
| Ca <sub>v</sub> 1.3 42a (short)             | -6.36 ± 0.02<br>N = 5-6         | 1.0 ± 0.05            | 91 ± 2             | ND                            | -40 ± 1.2<br>N = 5           | -28 ± 0.7<br>N = 5         | ND                                      |
| Ca <sub>v</sub> 1.3/DHPi                    | ~ -4.0<br>N = 2-16              | ND                    | ND                 | ND                            | -27 ± 1.2<br>N = 5           | -22 ± 1.1<br>N = 9         | -9.5 ± 1.4<br>N = 6                     |
| Ca <sub>v</sub> 1.3/MV                      | -7.05 ± 0.04<br>N = 5-7         | 1.3 ± 0.15            | 81 ± 2             | -6.11 ± 0.04<br>N = 5         | -35 ± 0.5<br>N = 6           | -26 ± 1.1<br>N = 23        | ND                                      |
| Ca <sub>v</sub> 1.2/VM                      | -7.41 ± 0.07<br>N = 4-6         | 0.83 ± 0.11           | 94 ± 4             | ND                            | -38 ± 0.5<br>N = 6           | -24 ± 1.0<br>N = 8         | ND                                      |
| Ca <sub>v</sub> 1.3+                        | -7.00 ± 0.02<br>N = 6-8         | 0.79 ± 0.02           | 87 ± 1             | ND                            | -38 ± 1.6<br>N = 7           | -29 ± 0.8<br>N = 2         | ND                                      |
| Ca <sub>v</sub> 1.3+V                       | -7.37 ± 0.05<br>N = 4-10        | 1.4 ± 0.16            | 83 ± 2             | -6.97 ± 0.05<br>N = 3-7       | -42 ± 0.3<br>N = 4           | -28 ± 1.2<br>N = 8         | -8.2 ± 1.8<br>N = 7                     |
| Ca <sub>v</sub> 1.3/PEEP                    | -6.73 ± 0.07<br>N = 3-7         | 0.43 ± 0.02           | 80 ± 2             | -5.93 ± 0.03<br>N = 4-5       | -36 ± 0.2<br>N = 3           | -27 ± 0.8<br>N = 6         | ND                                      |
| Ca <sub>v</sub> 1.3/N6                      | -6.93 ± 0.20*<br>N = 5-9        | 0.52 ± 0.12           | 66 ± 5             | -6.05 ± 0.15*<br>N = 8-12     | -34 ± 0.6<br>N = 5           | -17 ± 0.8<br>N = 9         | ND                                      |
| Ca <sub>v</sub> 1.3/SA                      | -7.01 ± 0.11<br>N = 4-5         | 0.82 ± 0.2            | 90 ± 5             | -6.01 ± 0.40<br>N = 3-18      | -49 ± 0.8<br>N = 12          | -29 ± 1.5<br>N = 12        | ND                                      |

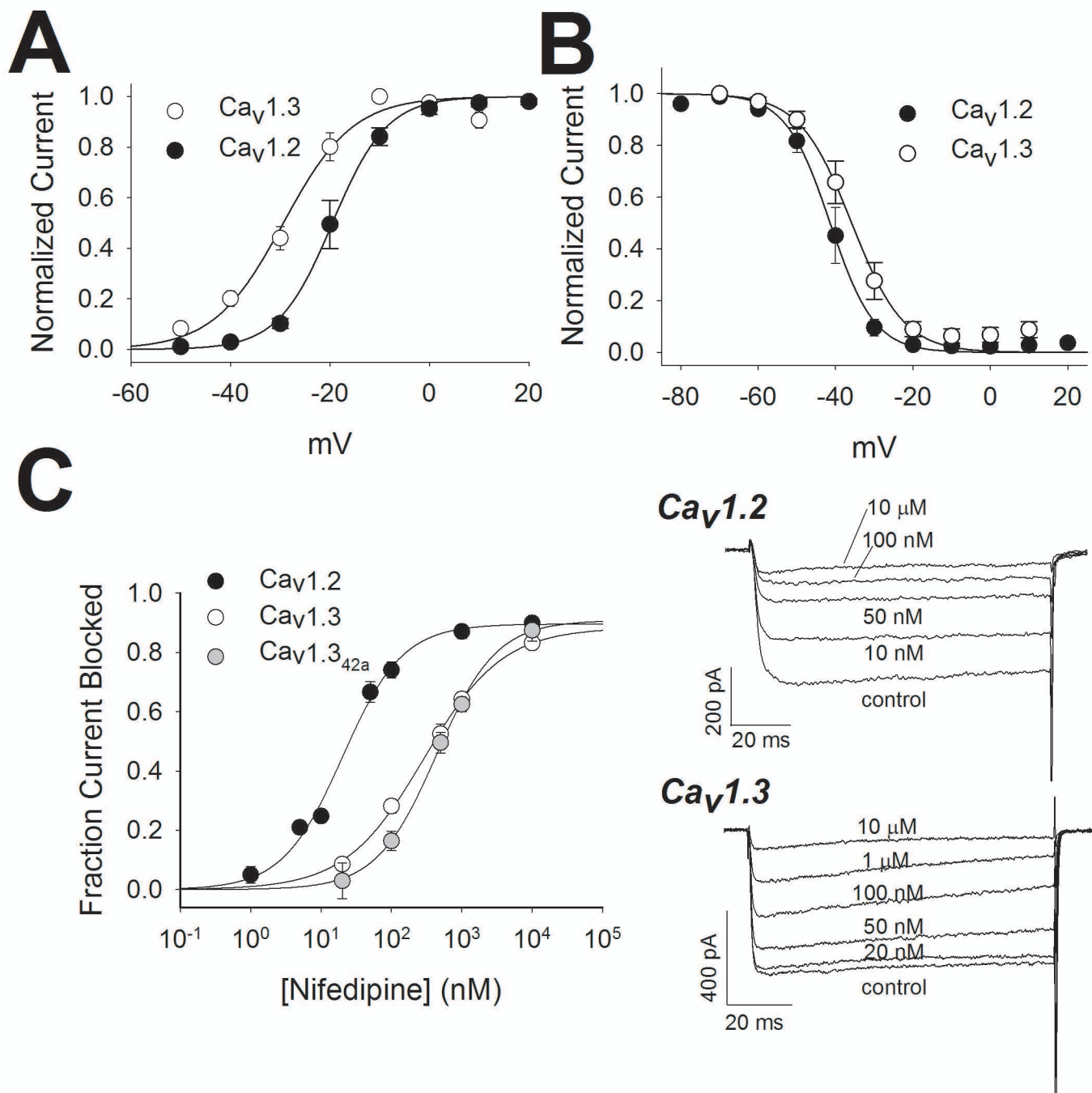
\*Data collected using balanced NMDG solutions, ND- Not Determined

**Table 2. Kinetics of Tail Current Decay in the Presence and Absence of FPL 64176**

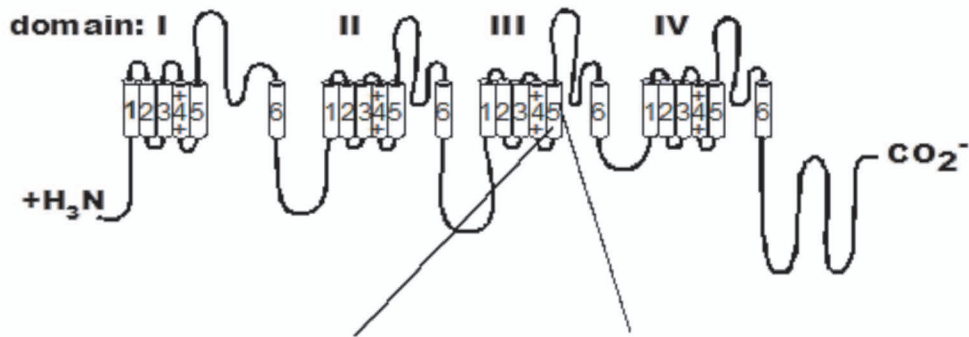
| Channel                  | Frac. Fast | $\tau$ -fast (msec)      | Frac Slow  | $\tau$ -slow (msec) | Frac Slow FPL | $\tau$ -FPL (msec)    | FPL R10                   | N |
|--------------------------|------------|--------------------------|------------|---------------------|---------------|-----------------------|---------------------------|---|
| Ca <sub>v</sub> 1.2      | 0.79 ± .08 | 0.37 ± .05               | 0.18 ± .08 | 6.5 ± .6            | 0.64 ± .20    | 24 ± 7 <sup>#</sup>   | 0.67 ± .09 <sup>***</sup> | 6 |
| Ca <sub>v</sub> 1.3      | 0.96 ± .01 | 0.41 ± .07               | NA         | NA                  | 0.34 ± .06    | 11 ± 1 <sup>###</sup> | 0.15 ± .02                | 5 |
| Ca <sub>v</sub> 1.3/DHPi | 0.94 ± .02 | 0.59 ± .11               | NA         | NA                  | NA            | 0.60 ± .04            | ND                        | 5 |
| Ca <sub>v</sub> 1.3+V    | 0.96 ± .01 | 0.70 ± .13               | NA         | NA                  | 0.84 ± .05    | 6.0 ± 1 <sup>##</sup> | 0.39 ± .07 <sup>*</sup>   | 6 |
| Ca <sub>v</sub> 1.3/MV   | 0.97 ± .01 | 0.71 ± .04 <sup>*</sup>  | NA         | NA                  | 0.53 ± .13    | 39 ± 9 <sup>#</sup>   | 0.52 ± .15 <sup>*</sup>   | 5 |
| Ca <sub>v</sub> 1.2/VM   | 0.96 ± .04 | 0.68 ± .15               | NA         | NA                  | 0.73 ± .10    | 29 ± 7 <sup>##</sup>  | 0.71 ± .08 <sup>***</sup> | 6 |
| Ca <sub>v</sub> 1.3/PEEP | 0.96 ± .02 | 0.77 ± .08 <sup>**</sup> | NA         | NA                  | 0.71 ± .18    | 12 ± 3 <sup>##</sup>  | 0.34 ± .11                | 5 |

<sup>\*</sup>,  $P < 0.05$ ; <sup>\*\*</sup>,  $P < 0.01$ ; <sup>\*\*\*</sup>,  $P < 0.001$  compared to Ca<sub>v</sub>1.3

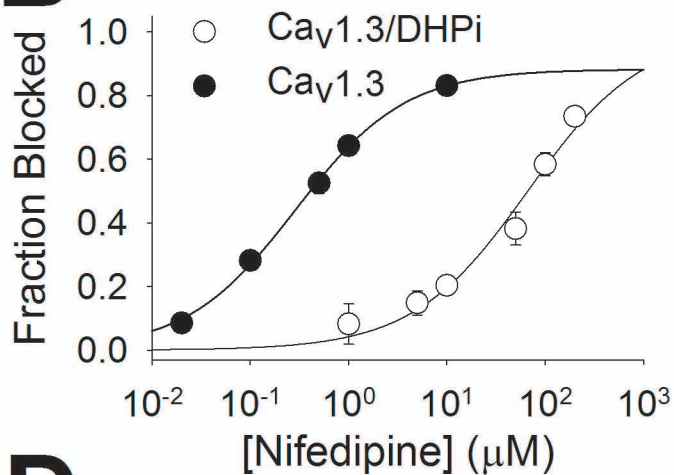
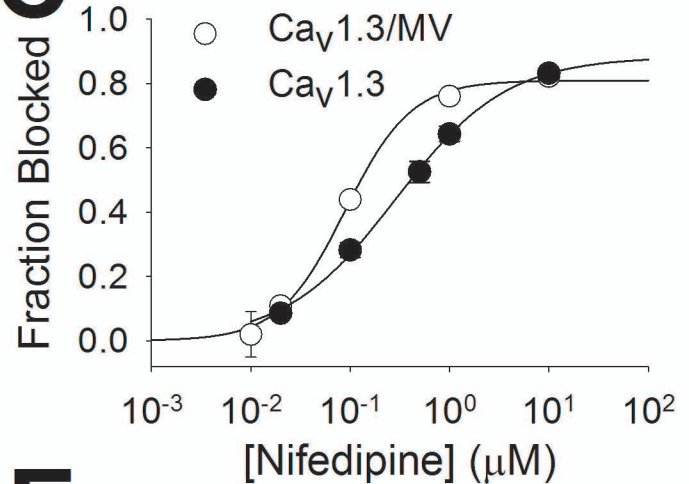
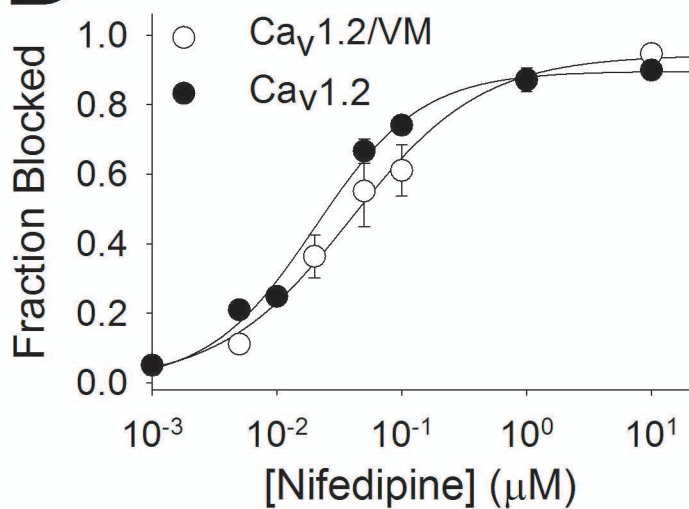
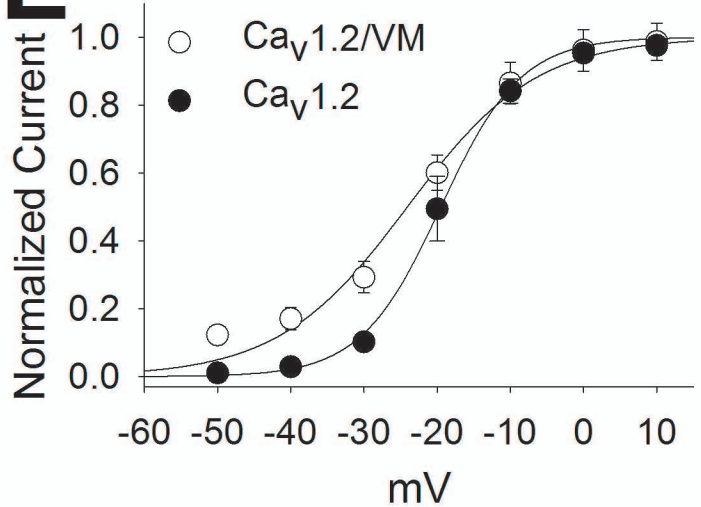
<sup>#</sup>,  $P < 0.05$ , <sup>##</sup>,  $P < 0.01$ , <sup>###</sup>,  $P < 0.001$  compared to absence of FPL



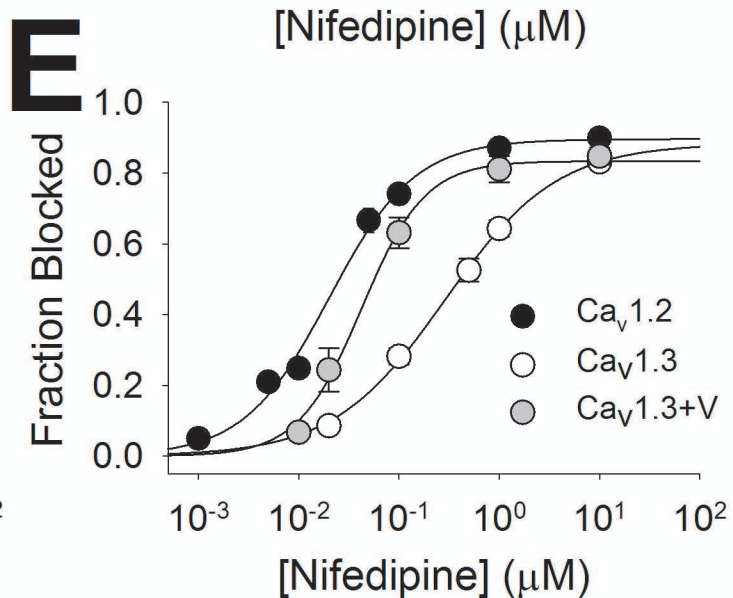
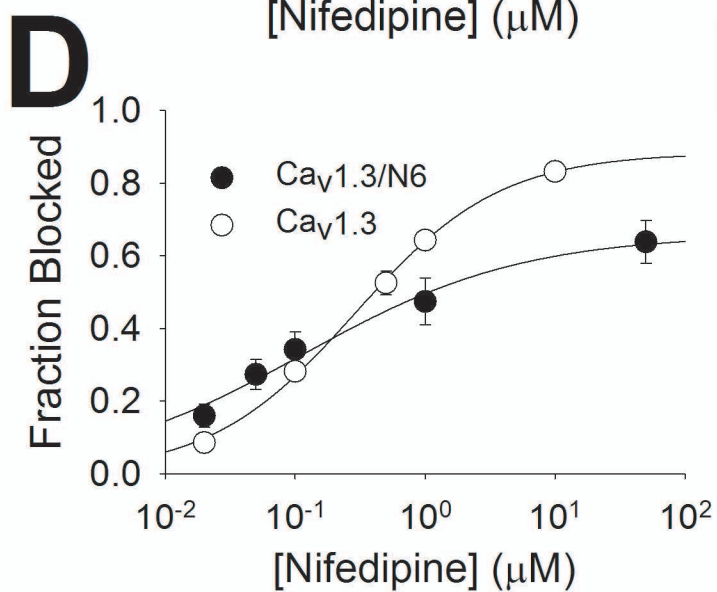
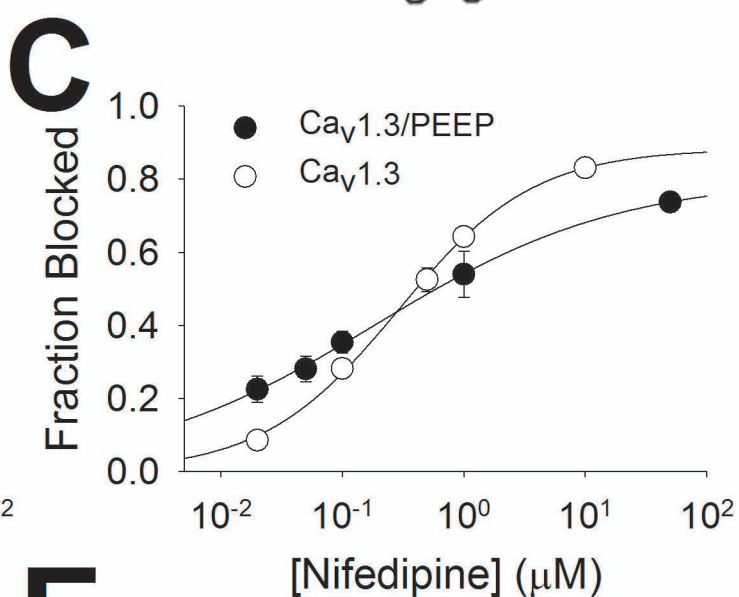
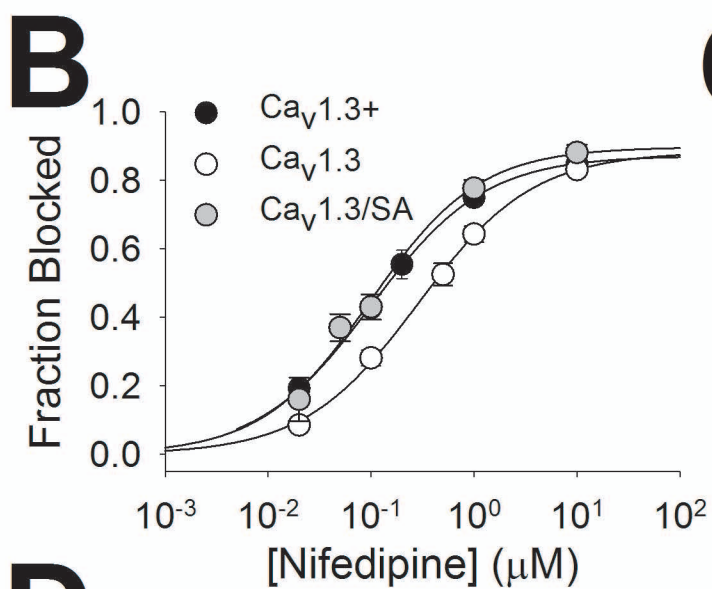
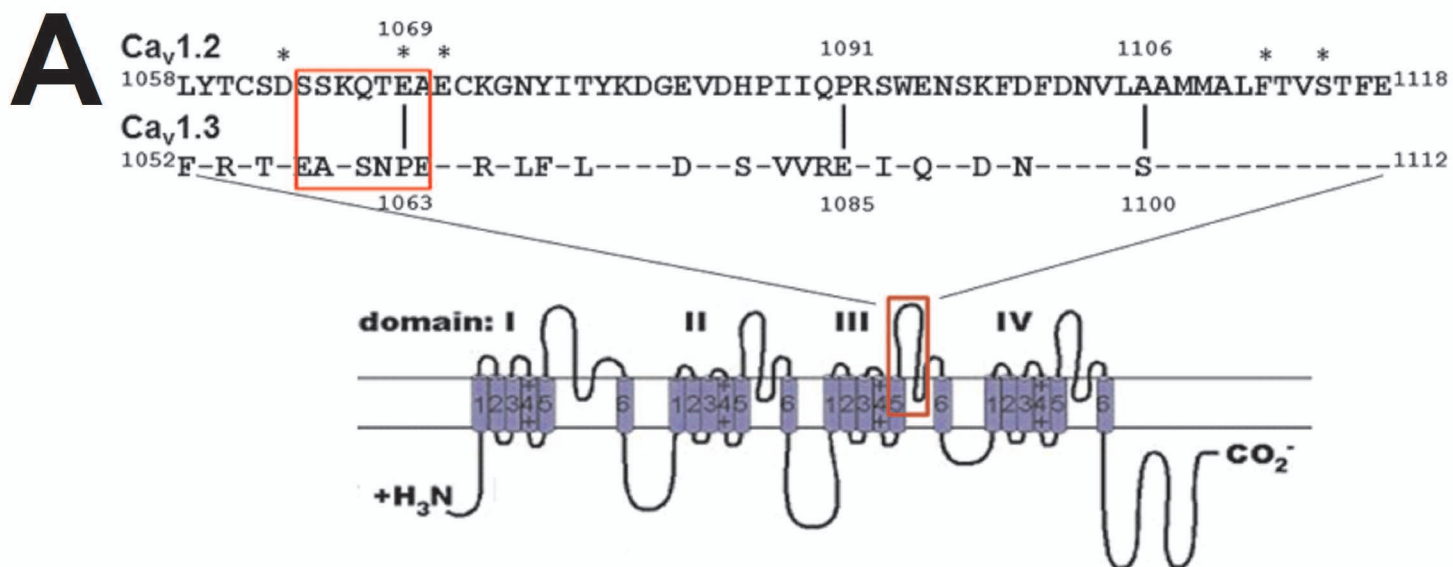
**Figure 1**

**A**

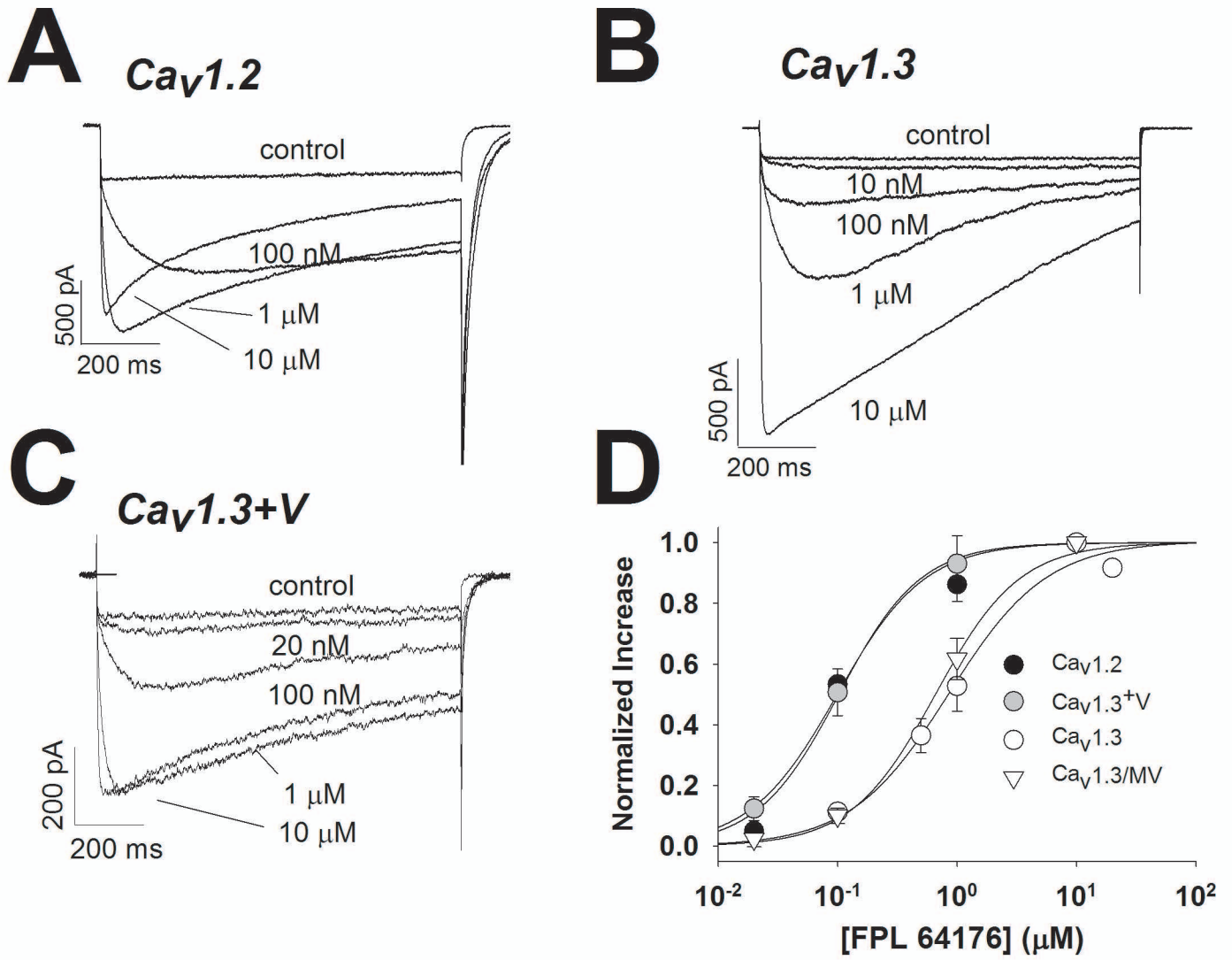
*Ca<sub>v</sub>1.3*            TIGNIMIVTLLQFMFACIGVQLF  
*Ca<sub>v</sub>1.3/DHP<sub>i</sub>*    -----Y-----M-----  
*Ca<sub>v</sub>1.2*            TIGNIVIVTLLQFMFACIGVQLF

**B****C****D****E****Figure 2**

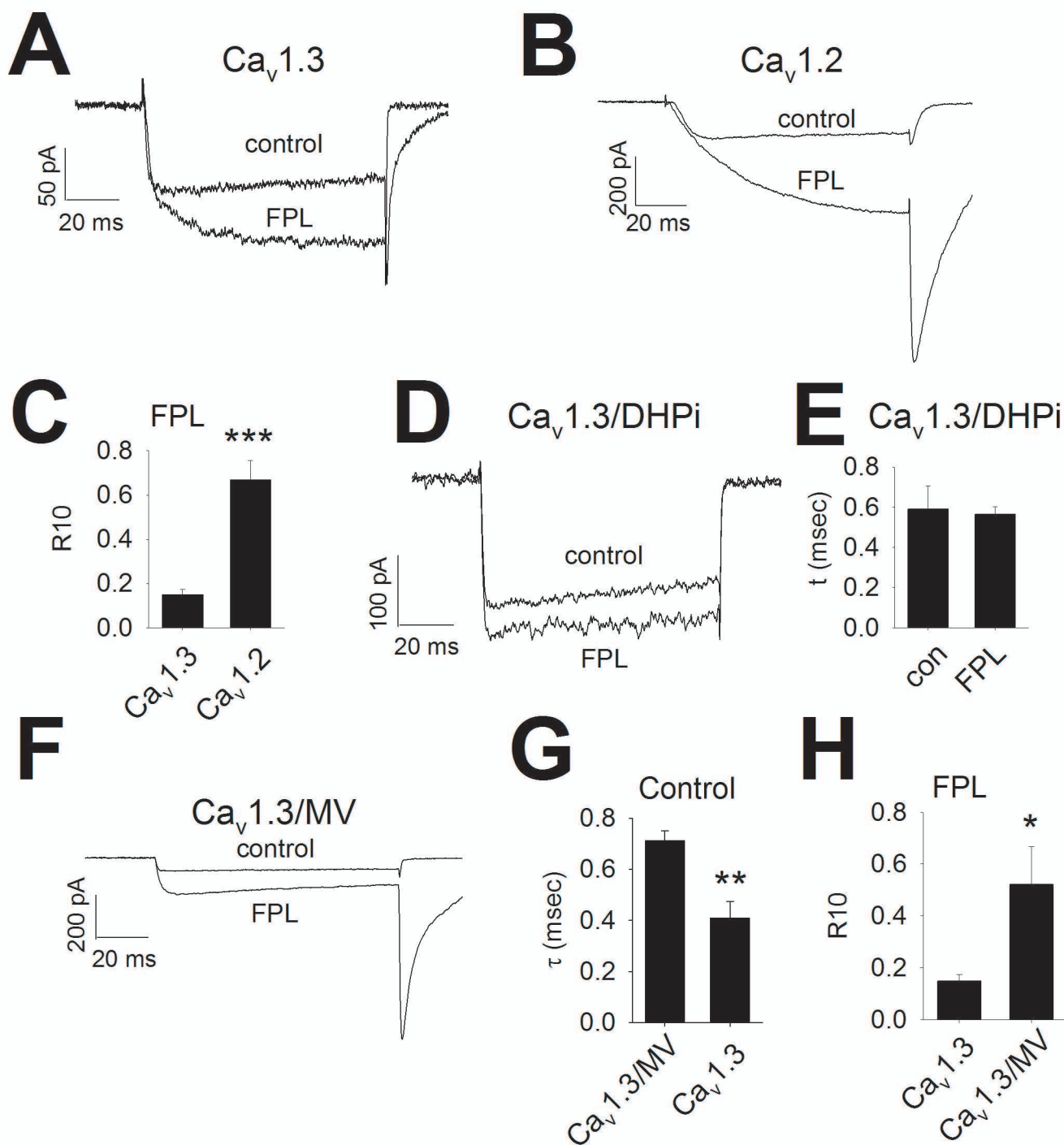




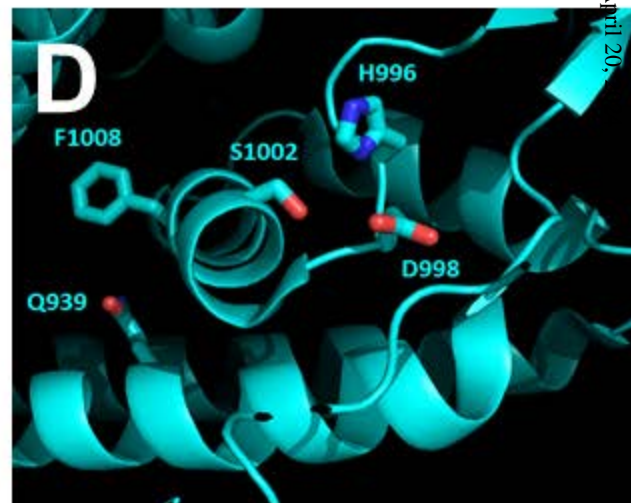
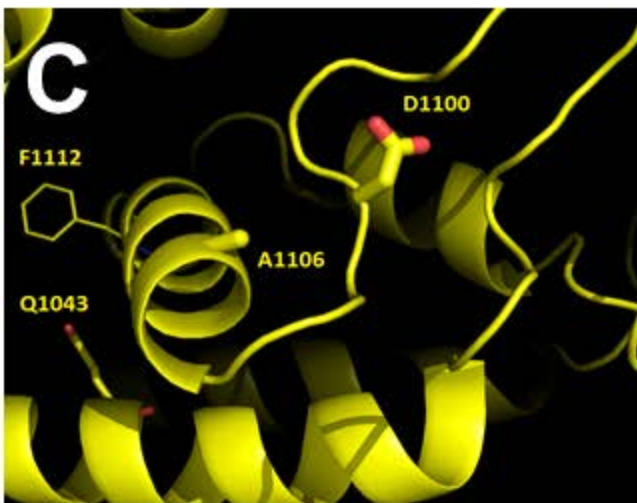
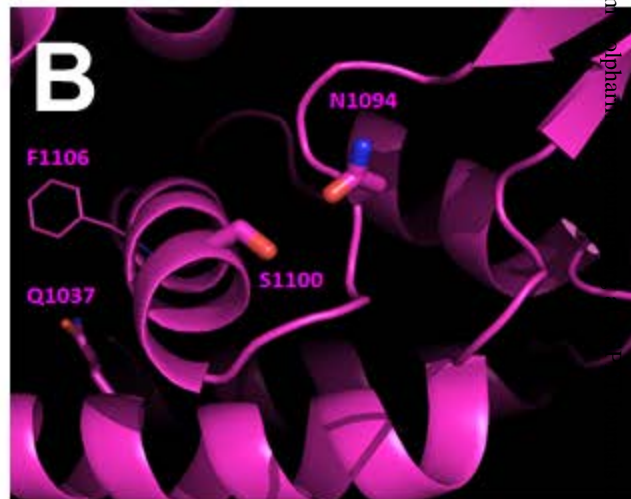
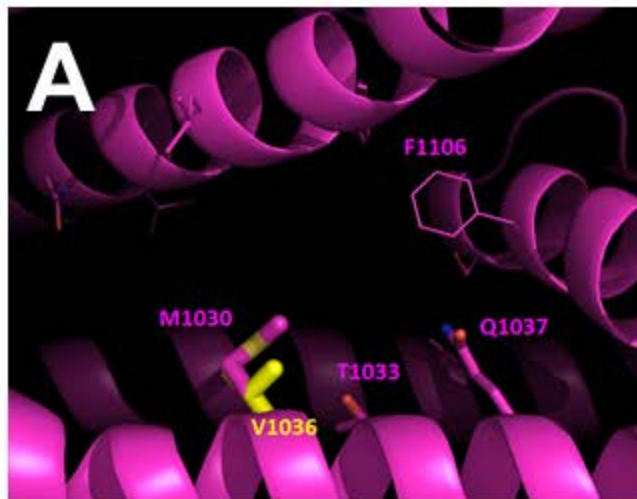
**Figure 3**



**Figure 4**



**Figure 5**



**Figure 6**