Mapping of dihydropyridine binding residues in a less sensitive invertebrate L-type calcium channel (LCaV 1)

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Abbreviations: DHP, 1,4-dihydropyridines

Invertebrate L-type calcium channel, LCaV 1, isolated from the pond snail Lymnaea stagnalis is nearly indistinguishable from mammalian CaV 1.2 (α1C) calcium channel in biophysical characteristics observed in vitro. These L-type channels are likely constrained within a narrow range of biophysical parameters to perform similar functions in the snail and mammalian cardiovascular systems. What distinguishes snail and mammalian L-type channels is a difference in dihydropyridine sensitivity; 100 nM isradipine exhibits a significant block of mammalian CaV 1.2 currents without effect on snail LCaV 1 currents. The native snail channel serves as a valuable surrogate for validating key residue differences identified from previous experimental and molecular modeling work. As predicted, three residue changes in LCaV 1 (N 3018, F 3110 and L 4112) replaced with D α 3.5.4-9 The resulting protein complexes included the primary, drug binding α1 subunit from skeletal Muscle, providing a greater abundance of Cav1.2 channels for this work. Subsequent swapping of amino acid sequences between DHP-sensitive Cav1.2 channels and a non-sensitive, mammalian surrogate, served to confirm that the regions necessary for DHP block included regions labeled by photoactive DHP of CaV1.1 channels.11-15,17-21 Identified through this work was a DHP binding region delimited to a unified and compact binding site in the CaV1.1 channel (N 3018, F 3110 and M 4112) raises the potency of isradipine block of LCaV1 channels to that of mammalian CaV1.2. Interestingly, the single N 3018_Q mutation in LCaV1 channels lowers DHP sensitivity even further and the triple mutation bearing enhanced isradipine sensitivity still retains a reduced potency of agonist, (S)-Bay K8644.

Introduction

The drug class of dihydropyridines (DHPs) are L-type calcium channel ligands that have been the subject of intense research efforts since their discovery by Fleckenstein, Godfraind and Polster over 30 years ago.1,2 L-type calcium channel blockers such as amlodipine are clinically relevant DHPs, and are amongst the most extensively used therapeutics worldwide for the treatment of hypertension and angina.1

DHPs are potent and selective blockers of α1C, CaV1.2, L-type calcium channels of the cardiovascular system (heart, blood vessels, conducting system).3 L-type channel complexes were isolated in classic biochemistry experiments from tissue extracts.4-9 The resulting protein complexes included the primary, drug binding α1 subunit from skeletal Muscle and additional auxiliary subunits (such as αδ and β) which are not directly involved in drug binding, but are necessary for promoting the expression of functional CaV1+ channels in vitro.10 Heterologous expression and analysis of recombinant channels has permitted the identification of relevant DHP binding pocket residues in CaV1.2. Much of the painstaking work took place in the 1990s and the early 2000s in several laboratories.1-23

The general locale for DHP binding was first identified by photoaffinity-labeling DHPs on proteolyzed fragments of CaV1.1 channels, which were immunoprecipitated with specific antibodies.13,18 DHP-sensitive, CaV1.1 channels derived from skeletal muscle, provided a greater abundance of CaV1.2 channels for this work. Subsequent swapping of amino acid sequences between DHP-sensitive CaV1.2 channels and a non-sensitive, mammalian surrogate, served to confirm that the regions necessary for DHP block included regions labeled by photoactive DHP of CaV1.1 channels.10-15,17-21 Identified through this work was a DHP binding region delimited to a unified and compact binding site in the CaV1.2 channel at the interface of domains III and IV.24 Together, experimental models establish the DHP binding region to a series of residues with close proximity to each other that make up 0.72% (or 16/2,220 amino acids) of CaV1.2 Ca2+ channel protein.

The large size and hydrophobic nature of CaV1.2 calcium channels has precluded their structural studies at the atomistic level by X-ray crystallography. The best alternative to date is homology modeling. A recent model by Tikhonov and Zhorov25 was built using Monte Carlo energy minimization of the pore-forming domain of CaV1.2, and is based on the crystal structure of prokaryotic K+ channel KAP in the open state.26 with DHP ligands docked to DHP-sensing residues known from experiments. The
Results

Structural similarity of L-type calcium channel from pond snail *Lymnaea stagnalis* (LCa1). Ca1 channels are distinguished by their sensitivity to 1,4 dihydropyridines (DHPs) and supporting a “Long lasting” or L-type Ba2+ current with large unitary conductance, with a pronounced Ca2+-dependent inactivation that was described in snail 27,28 and mammalian 29 neurons. There is only a singleton invertebrate L-type channel gene, whose product in the pond snail, *Lymnaea stagnalis* is dubbed “LCav1”. A speciation from an ancestral gene could have created the total of four Cav1, L-type calcium channel genes in mammals: Ca1.1 (α1S), Cav1.2 (α1C), Cav1.3 (α1D) and Cav1.4 (α1F), (Fig. 1A). An absence of more than one gene for L-type channels is supported in available data from completely sequenced invertebrate genomes to date (e.g., *C. elegans*, *D. melanogaster*, *S. purpuratus*). Ca1.2 channels are the primary drug target for 1,4 dihydropyridines in ventricular muscle or vascular smooth muscle30,31 and are structurally more similar to the other mammalian calcium channel genes (Ca1.1, Ca1.3 and Ca1.4) than to the more distant invertebrate gene from the pond snail (Fig. 1B). Regions of
high conservation between the invertebrate and Ca$_{1.2}$ homologs can be found in the six membrane-spanning segments of all four repeat domains (DI to DIV), and also in the cytoplasmic loops associated with accessory β subunit (Ca$_{1.2}$)-binding (AID or A-Interaction Domain) or the primary binding site for calmodulin-β subunit (Cav1 associated with accessory repeat domains (DI to DIV), and also in the cytoplasmic loops can be found in the six membrane-spanning segments of all four calmodulin-binding C-terminus of LCav1 at the Pre-IQ and IQ domain (Pre-IQ/IQ) associated with calcium-dependent inactivation (Fig. 1C). 

Snail LCav1 channel is almost indistinguishable from mammalian Ca$_{1.2}$ channel in biophysical properties. From a higher structural similarity amongst the mammalian L-type channel genes, it would be predicted that the more distant snail LCav1 channel would exhibit a hybrid of biophysical features of nine mammalian Ca$_{2+}$- channels subtypes. Surprisingly, this is not the case. The expressed currents of the snail LCav1 appear to be similar to those observed with a mammalian equivalent Ca$_{1.2}$ in whole-cell patch clamp recordings of transiently-transfected channels in human embryonic kidney (HEK-293T) cells with barium as a charge carrier (for detailed biophysical comparison between LCav1 and Ca$_{1.2}$, see Table 1). LCav1 is also similar to Ca$_{1.2}$ channels in possessing approximately two fold larger currents in barium than calcium, and a characteristic calcium-dependent inactivation with almost identical residues in the calmodulin-binding C-terminus of LCav1 in the Pre-IQ and IQ motifs. 

An ensemble of similar traces are revealed with voltage steps from a holding potential of -100 mV to more depolarized potentials (Fig. 2A) with sensitivities to voltage that are nearly identical for LCav1 and Ca$_{1.2}$, with a threshold at -30 mV, maximal currents at -10 mV in 15 mM barium external solution (Fig. 2B) and similar half-maximal activation of -10 mV when transformed using the Boltzmann equation (Fig. 2C).

A steady-state current is generated with a testing potential to peak current at +10 mV whose size is in proportion to the number of open and available channels in relation to inactivated ones after long, (5 s) conditioning pulses at different holding potentials (-100 to +40 mV) (Fig. 2D). The voltage-sensitivities of steady-state inactivation, like channel activation, are not distinguishable for snail and mammalian L-type channels, with 50% of inactivated channels at a similar ~20 mV steady-state potential extrapolated from Boltzmann-transformed curve fits (Fig. 2E).

Voltage steps to peak current sampled at a high rate in a shortened time base (35 ms) enables a closer examination of the rate of channel activation (Fig. 3A and sample traces, top). Running this protocol, a similar time to peak current were found in replicates for peak snail (LCav1) and mammalian (Ca$_{1.2}$) channel currents (Fig. 3A). A depolarizing +20 mV step extended for a longer period (20 s), allows for an analysis of the subsequent decay of current after channel opening and provides an estimate of the rate of channel inactivation. LCav1 and Ca$_{1.2}$ bear a similar decay rate indicated by their overlapping inactivation traces in a running window of mean ± SEM, in Figure 3B (top), or the similar current sizes at 50, 100, 500 and 1,000 ms time points (Fig. 3B and bottom). Decay times of LCav1 and Ca$_{1.2}$ currents are well fitted in a double exponential curve with similar fast ($\tau_1 = -200$ ms) and slow ($\tau_2 = -800$ ms) components. Replacing barium with calcium as the charge carrier, produces similar effects, such as a current that is -1/2 as large, reflecting a lower permeability of calcium ions compared to barium ions, and a faster inactivation rate, indicative of a calcium-dependent inactivation, a process that has been associated with pre-bound calmodulin to a highly conserved IQ motif in the C-terminus of invertebrate and mammalian L-type calcium channels. 

The rates at which channels recover from inactivation is measured by the size of current recovered at a hyperpolarized potential (-100 mV) for various time intervals (1 ms to 250 s) starting from a position where the channel has been held for a long period (20 s) at a depolarized +20 mV potential, that completely inactivates the channel currents. As illustrated in Figure 3C, Ca$_{1.2}$ and LCav1 channels have a similar temporal pattern of recovery from a state of complete inactivation, to one available to opening, with an approximate midpoint of recovery, which was respectively, 46.42% ± 8.01 compared to 56.64% ± 4.64 recovery at 1.783 s. Complete recovery from inactivation requires approximately 100 s for both channel types.

Illustrated in Table 1 is a summary of the biophysical comparisons for LCav1 and Ca$_{1.2}$. A remarkable similarity is found in side by side comparison of the two channels analyzed under the same conditions in HEK-293T cells, in spite of the

| Property          | Mammalian Ca$_{1.2}$ | Invertebrate LCav1 | LCav1 (N$^{354Q}$F$^{357Y}$I$^{452M}$) |
|-------------------|----------------------|--------------------|---------------------------------------|
| threshold$_{act}$ (mV) | -31.55 ± 0.59 (n = 20) | -29.34 ± 3.21 (n = 6) | -34.22 ± 5.21 (n = 4) |
| $V_{1/2, act}$ (mV)  | -2.10 ± 1.49 (n = 11)  | 0.43 ± 0.87 (n = 11)  | 1.20 ± 1.93 (n = 6)  |
| $V_{max}$ (mV)     | +13.49 ± 4.19 (n = 20) | +11.33 ± 4.15 (n = 6) | +14.33 ± 7.15 (n = 6) |
| $K_{act}$ (mV)     | 5.89 ± 0.6 (n = 11)    | 6.42 ± 0.33 (n = 11)  | 8.81 ± 1.10 (n = 6)  |
| Time to peak (ms)  | 15.05 ± 1.62 (n = 15) | 14.75 ± 1.38 (n = 16) | 13.90 ± 3.01 (n = 4) |
| $\tau_{act}$ (ms)  | 1.80 ± 0.079 (n = 11)  | 1.88 ± 0.083 (n = 7)  | 1.61 ± 0.230 (n = 4) |
| $V_{1/2, max}$ (mV) | -21.20 ± 1.89 (n = 11) | -22.32 ± 0.99 (n = 8)  | -19.32 ± 5.53 (n = 4) |
| $K_{inact}$ (mV)   | 5.77 ± 0.22* (n = 6)   | 7.48 ± 0.38* (n = 6)  | 7.11 ± 0.12 (n = 4)  |
| $\tau_{inact}$ (ms) | 204.37 ± 21.44 (n = 18) | 185.89 ± 16.34 (n = 11) | 319.20 ± 83.34* (n = 4) |
| $\tau_{inact2}$ (ms) | 829.35 ± 110.41 (n = 18) | 841.75 ± 65.26 (n = 11) | 1002.01 ± 35.20* (n = 4) |
| $\theta_{ind}$ recovery at 1.78 s | 56.64 ± 4.64 (n = 6) | 52.53 ± 4.42 (n = 6) | 46.42 ± 8.01 (n = 3) |

*significant difference (p < 0.05) in a One-Way Anova.
overall structural divergence of the snail channel compared to mammalian L-type channels (Fig. 1A and B).

LCa\textsubscript{1} has reduced affinity for DHP blocker, isradipine that is caused by three residue differences. Despite the overall similarities in the biophysical features between LCa\textsubscript{1} and Ca\textsubscript{1.2} channels, there is significantly reduced sensitivity of the snail channel for the DHP blocker isradipine compared to mammalian Ca\textsubscript{1.2} channel. A series of representative doses for isradipine are illustrated in Figure 4, where it is evident that 100 nM has more than a ¾ blocking effect of mammalian Ca\textsubscript{1.2} currents (Fig. 4B), while minimally affecting snail LCa\textsubscript{1} currents. The difference is a right-shifted dose response curve for LCa\textsubscript{1} (Fig. 4C) and an IC\textsubscript{50} that is 632 ± 152.5 nM, n = 5 (LCa\textsubscript{1}) and much higher than Ca\textsubscript{1.2} (43.2 ± 2.6 nM, n = 8) (Fig. 4C and inset). Although the snail channel requires a higher concentration of drug for equal effectiveness, the mode of drug block appears to be the same.\textsuperscript{34} Steady-state availability is shifted significantly to the left in the hyperpolarizing direction, in the presence of drug (IC\textsubscript{50} = -46.65 ± 2.63 nM (+isradipine), n = 6 versus -22.32 ± 0.99 nM, n = 8 (-isradipine)) indicative of a voltage-dependence of the drug block (Fig. 4D), as observed for Ca\textsubscript{1.2} channels.

Three residue differences between LCa\textsubscript{1} vs. Ca\textsubscript{1.2} are expected to alter DHP sensitivity. The primary drug-targeted

![Figure 2](https://example.com/f2.png)

**Figure 2.** Snail LCa\textsubscript{1} channel is indistinguishable from mammalian Ca\textsubscript{1.2} channel in voltage-dependence of gating. (A) Representative ensembles of voltage-sensitive barium currents for LCa\textsubscript{1} and Ca\textsubscript{1.2} channels generated using the voltage clamp protocol indicated in inset. (B) Ohmic-Boltzmann fitted current-voltage relationships also transformed into (C) activation curves with fitted Boltzmann equation for LCa\textsubscript{1} channels (n = 11) and Ca\textsubscript{1.2} channels (n = 6). Steady-state availability protocol (D) generates steady-state availability curves (E) for LCa\textsubscript{1} channels (n = 8) and Ca\textsubscript{1.2} channels (n = 11).
The α1 subunit consists of four repeats (I, II, III and IV) connected by cytoplasmic linkers. Each repeat consists of six transmembrane helices (S1 through S6), and contributes to a quadrant of the channel pore, with the pore-lining residues consisting of the ascending limbs in the extracellular membrane re-entering pore loops (P-loops) between S5 and S6 and the cytoplasmic two-thirds of S6 (Fig. 5A). Specific residues reported to be associated with DHP sensitivity are expected to be in close proximity to each other in the native channel (Fig. 5C) and are delimited to repeat III outer helix S5 (3o), the pore loops of repeats III and IV (3p and 4p) and the inner helices of repeats III and IV (3i and 4i) (Fig. 5B). A recent molecular model by Tikhonov and

**Figure 3.** Invertebrate LCaV1 is indistinguishable from mammalian Cav1.2 in rate kinetics. (A) (top) Overlapping of representative barium current traces at peak current for LCaV1 and Cav1.2 over shortened time base (35 ms) illustrating similar activation rate, measured in bar graph (below) as similarity in time to peak (mean ± SEM) current for LCaV1 (n = 16), Cav1.2 (n = 15) channels. Peak currents were generated at +10 mV from a holding potential of -100 mV. (B) (top) Longer 2 s time base illustrates the rate of decay (shown as mean ± SEM) of LCaV1 (n = 11), Cav1.2 (n = 18), also measured (below) as fraction of peak current (I/I_max) at 50 ms, 100 ms, 500 ms, 1000 ms time points. (C) Log plot of the recovery from inactivation rate measured by the size of current recovered at a hyperpolarized potential (-100 mV) for various time intervals (0.56 ms to 250 s) starting from a completely inactivated state. The approximate midpoint of recovery from inactivation for both channel types was 1.8 s.
significant contributions to the DHP-channel binding energy (green residues, Fig. 5B); the latter residues may have allosteric effects on drug binding.

The homology model suggests that the DHP potency in Ca\textsubscript{1.2} depends on the ligand-channel H-bonds involving side
Figure 5. Previous experimental data and modeling suggest three residues difference in the snail channel that can alter DHP sensitivity of the snail channel. (A) Photoaffinity labeling, chimera and site-directed mutagenesis work delimited the dihydropyridine binding to blue highlighted regions illustrated in channel structure diagram: Domain III, segment 5 (3o)—pore loop (3p)—segment 6 (3i) and Domain IV, pore loop (4p)—segment 6 (4i). (B) Three residue differences (red color) in snail channel (N 3o18Q, F3i10Y, I4i12M) out of 15 identified by Tikhonov and Zhorov model (red and blue colors) in amino acid alignment of LCa 1 and Ca 1.2. (C) Cytoplasmic and pore views of (R)-isradipine docked in Ca 1.2 as in the model by Tikhonov and Zhorov. The DHP ligand is shown by sticks. DHP-sensing residues are space filled and hydrogen atoms are omitted for clarity. Ca 2+ ions in the selectivity filter are shown as yellow spheres.
chains of Q₁₃₀₁₈, Y₁₃₁₀ and Y₁₄₁₁. The α-carbons of these residues form a plane, which also accommodates the innermost of two calcium ions in the outer pore. The inflexible, boat-shaped DHP molecule contains three H-bonding counterparts: (Figs. 5C and 9A) the invariant NH group at the stern which binds to Y₁₃₁₀ in IIIS₆, a polar group at the bowsprit accepting an H-bond from Y₁₄₁₁ in IVS₆ and a carbonyl oxygen at the starboard side accepting an H-bond from Q₁₃₀₁₈ in IIIS₅ (Figs. 5C and 9A). Also playing a role in the starboard side is a hydrophobic pocket formed by residues that include M₄₁₂ in IVS₆ (Figs. 5C and 9A).

Both modeling and experimental data suggest that three substitutions in the mammalian Caᵥ₁.₂ channel would decrease the DHP sensitivity similar to that of LCaᵥ₁: Q₃₀₁₈N, Y₃₁₀F and M₄₁₂I. Previous experimental data collected using placement of residues on mammalian surrogate channels, such as Caᵥ₂.₁, suggest that changes to any of these residues in the Caᵥ₁.₂ markedly affect dihydropyridine binding.¹¹⁻²³

A triple mutant in LCav₁, in which the key residue differences with Caᵥ₁.₂ (N₃₀₁₈Q, F₃₁₀Y and I₄₁₂M) identified in modeling and previous experimental data are eliminated, has sensitivity to the DHP blocker isradipine comparable to that of Cav₁.₂. The triple mutant is almost completely blocked by isradipine at a 100 nM dose, which is a concentration that was almost without effect for wild-type LCaᵥ₁ channels (Fig. 6A). The dose response curves shifts to one that overlaps with Caᵥ₁.₂ (Fig. 6B) with an IC₅₀ of the triple mutant (64.5 ± 9.8 nM, n = 7) similar to Caᵥ₁.₂ (43.2 ± 2.6 nM, n = 8; Fig. 6C). The remarkable outcome of the triple mutant is a snail channel that is not easily separated by biophysical or pharmacological means from the human homolog. An enhanced blocking capacity of the triple mutant was not the result of changes in biophysical parameters, since these did not differ from wild-type LCav₁ channels (Table 1).

Individual mutations are not equal in their contribution to antagonism to isradipine. Individual residue differences are not equal in their contribution to effect of isradipine. (IC₅₀ values for LCaᵥ₁, Caᵥ₁.₂ and LCaᵥ₁ mutants are shown in Table 2). Single mutants (F₃₁₀Y or I₄₁₂M) add to the potency of the DHP blocker (Fig. 7A and B and Table 2), while the single mutant N₃₀₁₈Q, surprisingly, causes a loss in sensitivity to blockade even when compared to the wild-type LCav₁ channel. Other amino acid replacements at the 3₀₁₈ site were created to investigate what side chain property of glutamine (Q) were responsible for worsening the sensitivity of LCav₁ for isradipine compared to asparagine (N). Shortening the side chain with either non-polar, alanine or polar, serine with an H-bonding side chain (N₃₀₁₈A, N₃₀₁₈S), were equivalent in DHP blockade to asparagine (N) at that position, suggesting that the added length of a carbon side chain of glutamine (Q) at N₃₀₁₈Q, provides a hindrance to drug binding, which was not apparent in the double or triple mutants of LCav₁ (Fig. 7C and D and Table 2). A large substituent lysine (N₃₀₁₈K) did not form expressible channels. Potency of the double mutants appears additive based on their individual contributions in the single mutants (Fig. 7E and F and Table 2). A combination of all three I₄₁₂M/ F₃₁₀Y/N₃₀₁₈Q residue differences, however, is required for the full DHP potency equivalent to the Cav₁.₂ channel (Fig. 6).

Critical residue differences responsible for DHP antagonism are not key for the agonist’s potency. Core constituents of the
Figure 7. Individual mutants are not equal in their contribution to blockade to isradipine. Single mutants (A and B) and double mutants (E and F) in LCa\textsubscript{v}1 with residue changes N\textsuperscript{3018}Q, F\textsuperscript{3110}Y, I\textsuperscript{4112}M, generally show additive increases to antagonist DHP sensitivity from LCa\textsubscript{v}1 to Ca\textsubscript{v}1.2, with the exception of N\textsuperscript{3018}Q, which lowers antagonist DHP sensitivity substantially. (C and D) Comparison of single mutants at 3\textsuperscript{o}18: N\textsuperscript{3018}A, N\textsuperscript{3018}S with the N\textsuperscript{3018}Q. (A, C and E) Dose response curves (mean ± SEM)(B, D and F) Extrapolated values for 50% blocking concentration (nM) illustrated in bar graphs.
drug-binding pocket are shared between DHP blockers such as isradipine and agonist DHPs such as (S)-(−)-Bay K8644. The snail channel is similar to Ca\textsubscript{1.2} channel having an enhanced whole cell current in the presence of maximal doses of the agonist (S)-(−)-Bay K8644 in the micromolar range (Fig. 8A). But lower doses reveal that LC\textsubscript{A1} is less sensitive to (S)-(−)-Bay K8644, which is consistent with reduced sensitivity of this channel to other DHPs such as the blocker isradipine (Fig. 8A). A threshold potency of 300 nM (S)-(−)-Bay K8644 produced a maximal increase in current, ~2.5-fold for mammalian Ca\textsubscript{1.2} channels (Fig. 8B). However, LC\textsubscript{A1} channels was almost non-responsive to 300 nM (S)-(−)-Bay K8644, while gradually increasing to a maximal current size averaging ~2-fold with a 5 μM dose. There is also an apparent marked increase in the rate of current decay for the snail channels, likely due to inactivation in the presence of the drug during prolonged depolarization at peak current (+10 mV) (Fig. 8A). The snail channel is also more sensitive to opening at low voltages as a result of a hyperpolarizing shift in the current-voltage relationship in the present of the agonist (S)-(−)-Bay K8644 (Fig. 8C). We also observe a reduction in the size of current enhancement for Bay K8644 at a holding potential of -60 mV versus -80 mV (data not shown). These data are consistent with models that suggest a voltage- and state-dependence for Bay K8644 binding to snail LC\textsubscript{A1} channels,\textsuperscript{36} although at weaker binding affinity. A similar Bay K8644 phenotype between snail and mammalian channels is suggestive of generally similar mechanisms for ligand action. However, the three residue changes of LC\textsubscript{A1} that increase the channel sensitivity to the DHP blocker (N\textsuperscript{3O18}Q, F\textsuperscript{3i10}Y and I\textsuperscript{4i12}M) do not increase potency of the agonist DHP (Fig. 8B).

### Discussion

The invertebrate L-type channel is a direct relative in the Ca\textsubscript{1} family to Ca\textsubscript{1.2}, exclusively, and is not easily discriminated from Ca\textsubscript{1.2} based on measureable parameters in vitro, under the same recording conditions. Such similarity is not evident in the overall amino acid sequences of the invertebrate Ca\textsubscript{1} channel and mammalian L-type calcium channels (Fig. 1A and B). Structural divergence reflects drift in amino acid sequences in the snail and mammalian isoforms over hundreds of millions of years since their speciation. These changes have largely occurred outside the invariant core of Ca\textsubscript{1} channels such as the membrane-spanning domains and the islands of conservation in cytoplasmic structures (e.g., Ca\textsubscript{1β} subunit and calmodulin binding; Fig. 1C).\textsuperscript{32} LC\textsubscript{A1} is the closest functional homologue to Ca\textsubscript{1.2} described to date, and perhaps this is not surprising, given their similar roles in the cardiovascular systems of snails\textsuperscript{37} and mammals\textsuperscript{38} and the likely specialized requirements in these tissues for their operation within a restricted range of parameters. Similarities between LC\textsubscript{A1} and Ca\textsubscript{1.2} include: (a) current-voltage relationships for activation and inactivation (Fig. 2); (b) “long-lasting” currents, with a pronounced Ca\textsuperscript{2+}-dependent inactivation in standard buffers with less voltage-dependent inactivation;\textsuperscript{35} (c) activation which is slow (Fig. 3) and requires a strong, high threshold depolarization to “open” the channel (Fig. 2); (d) ~2x larger current size when Ba\textsuperscript{2+} is the charge carrier instead of Ca\textsuperscript{2+} and (e) blockade of currents in micromolar Cd\textsuperscript{2+} concentration.\textsuperscript{33}

LC\textsubscript{A1} is a relevant surrogate to examine the structure and function of DHP blockade. Invertebrates have only a single L-type calcium channel gene, and may not require the structural diversity in L-type calcium channel genes such as Ca\textsubscript{1.1}, Ca\textsubscript{1.3} or Ca\textsubscript{1.4}. Alternative splicing of the invertebrate gene may provide some structural flexibility, but the absence of more than one gene likely relates to lack of functional requirements. Invertebrates lack organized striated muscle in tetrads and a direct coupling of a calcium channel to ryanodine receptors of the sarcoplasmic reticulum, where Ca\textsubscript{1.1} channels have an exclusive function.\textsuperscript{39} Ca\textsubscript{1.4} channels are associated with ciliary photoreception in vertebrates, whereas a completely different rhabdomeric mechanism occurs in invertebrates, transduced by light-activated depolarizing, not hyperpolarizing potentials, mediated by unique calcium-selective, Trp channels.\textsuperscript{40} Outside of muscle and the retina, relevant mammalian cells contain either or both Ca\textsubscript{1.2} and Ca\textsubscript{1.3}.\textsuperscript{41} Expression of Ca\textsubscript{1.3} provides a wider coverage in the hyperpolarizing range and may be necessary for fast-acting, subthreshold depolarizations, such as to drive oscillatory activity and pulsatile release of hormones.\textsuperscript{45} An equivalent to Ca\textsubscript{1.3} may

### Table 2. Summary of sensitivities of L-type calcium channels (snail, snail mutants and mammalian) to isradipine

| Gene     | Mutation       | IC\textsubscript{50} (nM) | SEM     | n  | p < 0.05 | p < 0.005 |
|----------|----------------|--------------------------|---------|----|----------|----------|
| LCa\textsubscript{1} | none           | 632.2                    | 152.5   | 5  | *        |          |
| LCa\textsubscript{1} | N\textsuperscript{3O18}Q | 1304.2                  | 95.1    | 7  |          |          |
| LCa\textsubscript{1} | N\textsuperscript{3O18}A | 695.3                   | 121.2   | 4  | *        |          |
| LCa\textsubscript{1} | N\textsuperscript{3O18}S | 604.3                   | 130.2   | 4  | *        |          |
| LCa\textsubscript{1} | F\textsuperscript{3i10}Y | 281.3                   | 19.3    | 5  | *        |          |
| LCa\textsubscript{1} | I\textsuperscript{4i12}M | 449.8                   | 80.1    | 5  | *        |          |
| LCa\textsubscript{1} | N\textsuperscript{3O18}Q, F\textsuperscript{3i10}Y | 87.6      | 12.8    | 6  | *        |          |
| LCa\textsubscript{1} | N\textsuperscript{3O18}Q, I\textsuperscript{4i12}M | 293.3    | 81.1    | 5  | *        |          |
| LCa\textsubscript{1} | F\textsuperscript{3i10}Y, I\textsuperscript{4i12}M | 100.0     | 18.6    | 5  | *        |          |
| LCa\textsubscript{1} | N\textsuperscript{3O18}Q, F\textsuperscript{3i10}Y, I\textsuperscript{4i12}M | 64.5      | 9.8     | 7  | *        |          |
| Ca\textsubscript{1.2} | none           | 43.2                    | 2.6     | 8  |          |          |

Fig. 8B

Fig. 8C

Δ Ca\textsubscript{1.2}}
Figure 8. Invertebrate LCav1 has a reduced potency to agonist (S)-(-)-Bay K8644. (A) Representative enhancement of LCav1 and Cav1.2 barium currents in the presence of differing concentrations (0, 100, 300, 1,000, 5,000 nM) of agonist (S)-Bay K8644. Peak currents were generated at +10 mV from a holding potential of -80 mV. (B) Bar graph illustrating the effect of agonist (S)-Bay K8644, mean ± SEM (n = 4) on Cav1.2 channels and LCav1 channels and its triple mutant N318Q, F310Y, I412M. (C) Current-voltage relationships indicate a hyperpolarizing shift with the half-activation potential of LCav1 channel in the presence of 5 μM agonist Bay K8644 drug (n = 4).

not be necessary in invertebrates, where functions could overlap with other channels, such as Ca3 or T-type calcium channels.

Variations in DHP sensitivity have been reported amongst mammalian L-type calcium channels, although it is not straightforward to compare their sensitivities, since DHP block is highly dependent on the channel state. Operating voltages for Ca1.3 and Ca1.4 are shifted (15 mV) significantly to the hyperpolarizing range, which means that differences in DHP block do not reflect DHP affinity per se, but can be attributed to differences in voltage-sensitivity between the Ca1.2 and Ca1.3/1.4 channels. Variations in DHP sensitivity have been reported amongst mammalian L-type calcium channels, although it is not straightforward to compare their sensitivities, since DHP block is highly dependent on the channel state. Operating voltages for Ca1.3 and Ca1.4 are shifted (15 mV) significantly to the hyperpolarizing range, which means that differences in DHP block do not reflect DHP affinity per se, but can be attributed to differences in voltage-sensitivity between the Ca1.2 and Ca1.3/1.4 channels.41-44 It is not possible to attribute the low sensitivity in DHP block of the snail L-type channel to biophysical features, since these do not apparently differ much between the snail channel and Ca1.2. These preliminary findings lead us to hypothesize that there are critical differences for DHP activity that can be exploited using the snail channel as a surrogate for mutagenesis studies.

DHP-LCav1 interactions in view of the DHP-Ca1.2 model. Amino acids responsible for DHP binding were discovered in detailed mutagenesis studies of mammalian channels, through the late 1990s to early 2000s. A recent molecular model by Tikhonov and Zhorov is largely consistent with these earlier experimental findings.25 Validation of the modeling outcomes is particularly relevant, given that the three-dimensional structure of calcium channels is unknown and the homology model is built based on the x-ray structure of a distant voltage-gated potassium channel KAP.26 DHPs bind in the interface between repeats III and IV and both calcium channel blockers and agonists form H-bonds with residues in helices III5, III6 and IV56 in the homology model. The DHP portside faces into the pore lumen where calcium ions flow through the selectivity filter formed by glutamates from the four repeats: E1p50, E2p50, E3p50, E4p50.45 DHP blockers have hydrophobic side groups at their portside, which prevents the occupancy of the innermost binding site for calcium ions in the selectivity filter, whereas hydrophilic portside groups of agonists do not interfere with Ca2+ flow through the selectivity filter.

Confirmation of predicted residues responsible for reduced DHP blocker affinity in LCav1. The starboard substituent in a DHP ligand interacts with hydrophobic residues M412, I414, I418, amphiphilic T413 and hydrophilic Q418 according to the model (Fig. 8A). The substitution of flexible M412 in Ca1.2 with inflexible, bulky, β-branched isoleucine I412 in LCav1 would tighten the binding site for the starboard substituent (Fig. 8B and C) and decrease the potency of isradipine. Point mutations F410Y and I412M increase LCav1 sensitivity for isradipine by
providing an additional ligand-channel H-bond and widening the repeat interface, respectively, whereas the double mutation F1010Y/F1121M brings LCa\textsubscript{1} sensitivity to isradipine almost to the level of Ca\textsubscript{1.2}. Intriguingly, the point mutation N\textsuperscript{3018}Q in LCa\textsubscript{1} decreases rather than increases isradipine potency. This mutation effect can be explained because the mutation brings an additional methylene group to the already tight III/IV interface of LCa\textsubscript{1}. Predictably amino acids bearing short side chains with alanine or serine (such as in N\textsuperscript{3018}A, N\textsuperscript{3018}S) bear a potency similar when asparagine (N) is at this position.

Residue differences that are responsible for DHP blockade do not alter the potency of the agonist. Snail LCa\textsubscript{1} channels are significantly less sensitive to blocker isradipine compared to human Ca\textsubscript{1.2} channels, which is consistent with a reduced potency of the agonist (S)-Bay K8644. However, the three residue changes that contribute to the differences in affinity of the blocker do not alter the weaker potency of the agonist DHP in LCa\textsubscript{1} channels (Fig. 8B).

Absence of equal agonist sensitivity in the snail channel in the presence of high affinity DHP blockade in the triple mutant (N\textsuperscript{3018}Q, F\textsuperscript{3109}Y and F\textsuperscript{412}M) of the LCa\textsubscript{1} channel is consistent with findings in previous experimental studies using distantly-related and DHP-insensitive mammalian surrogates, from the non-L type, Ca\textsubscript{2.1} channel class. Swapping of eight or nine residue changes onto Ca\textsubscript{2.1} channel, dubbed \( \alpha\textsubscript{\text{Ca}2.1} \), creates a channel that is even more sensitive to DHPs than native Ca\textsubscript{1.2} channels.\textsuperscript{18,19,21} Mutations in homologous positions to increase DHP effects in snail LCa\textsubscript{1} channel at N\textsuperscript{3018}Q, F\textsuperscript{1010}Y and F\textsuperscript{412}M, reverses the DHP sensitivity in the mutated gen 2.1 channel, \( \alpha\textsubscript{\text{Ca}2.1} \), DHP (Q\textsuperscript{3018}N, Y\textsuperscript{3109}A and M\textsuperscript{412}M). In particular, any of these mutations in \( \alpha\textsubscript{\text{Ca}2.1} \) DHP eliminates agonist (Bay K8644) activity, while retaining varying degrees of the blocker (isradipine) phenotype.\textsuperscript{14,19-21,46}

The homology model is not precise enough to rationalize the free-energy energy increase through quantitative analysis of energetics of ligand-channel interactions. Nevertheless, considerations of ligand-channel contacts in the model suggest a possible explanation for the difference in agonist and blocker sensitivities for the snail channel. The 15-fold reduction of sensitivity to isradipine (Fig. 6C) corresponds to the 2.1 kcal/mol increase of free energy of the ligand-channel complex. LCa\textsubscript{1} is expected to provide a different set of residues for DHP binding as compared to the models of DHP-bound Ca\textsubscript{1.2}.\textsuperscript{25} First, LCa\textsubscript{1} lacks a hydroxyl group at F\textsuperscript{310} resulting in an absence of an H-bond with the DHP stern. This may be compensated by favorable electrostatic interactions (with possible H-bonding) between the DHP bow and S\textsuperscript{415}, in position where Ca\textsubscript{1.2} has A\textsuperscript{415}. Second, N\textsuperscript{3018} in LCa\textsubscript{1} is shorter than Q\textsuperscript{3018} in Ca\textsubscript{1.2} and would not reach the DHP starboard carbonyl oxygen as proposed for Ca\textsubscript{1.2}. Substituents in the bow aromatic ring of (S)-Bay K8644 and isradipine have very different geometry and the chemical nature of electronegative atoms (Fig. 9A). Fluorine atoms in Bay K8644 are weak H-bond acceptors\textsuperscript{82} and the rotatable CF\textsubscript{2} group of Bay K8644 is more adjustable to the receptor than H-bond acceptors in the benzoxadiazol ring of isradipine. Small and flexible (S)-Bay K8644 would more easily adjust to the DHP receptor in LCa\textsubscript{1} and establish an H-bond with T\textsuperscript{604}, a known DHP-sensing residue nearby N\textsuperscript{5018}. The fact that (S)-Bay K8644 has almost identical potencies in LCa\textsubscript{1} and its triple mutant (Fig. 8B) suggests that the small, adjustable agonist molecule establishes similar contacts in these channels. Such adjustments may be less likely for isradipine with its bigger, more rigid bow and the large isopropyl group. The above considerations are applicable to both enantiomers of isradipine. (R)-isradipine would be even less adjustable to the channel protein because the bulky isopropyl group at the starboard side would not fit as easily between I\textsuperscript{412} and I\textsuperscript{418} as the small methyl group at the starboard of (S)-Bay K8644 (Fig. 9C).

Caution is warranted in using surrogates for drug affinity studies. Use of a distantly related channel can juxtapose uniquely native residues contributing with the supplemented residues to support de novo high affinity DHP binding. The resulting artificially crafted hybrids may not reflect the true nature of the DHP binding in native L-type calcium channels. Consistent with this hypothesis, \( \alpha\textsubscript{\text{Ca}2.1} \) DHP mutant channels do not contain the state-dependent blockade characteristic of DHPs, like the native snail LCa\textsubscript{1}, whereby the affinity for block is significantly enhanced in the depolarized state.\textsuperscript{48} A state dependence of DHP blockade is a critical feature for its tissue selectivity in situ, contributing to the higher vascular selectivity of LTCCs seen with DHPs.\textsuperscript{48} Also some speculate that the higher affinity corresponds to the very different kinetic differences in Ca\textsubscript{2.1} and \( \alpha\textsubscript{\text{Ca}2.1} \) DHP, such as a very rapid inactivation compared to wild-type Ca\textsubscript{1.2}.\textsuperscript{24,42,43}

**Conclusion.** A native channel, LCa\textsubscript{1} from the snail, *Lymnaea stagnalis* bears indistinguishable biophysical properties as mammalian Ca\textsubscript{1.2} channels, and participates in similar cardiovascular functions. A critical difference between snail and mammalian channels is a ~15-fold lower potency to dihydropyridine block. We discuss how the drug characteristics for the native snail LCa\textsubscript{1} channel can be interpreted by the model described by Tikhonov and Zhorov,\textsuperscript{25} including possible differences in binding residues of the native snail channel that may influence agonist and blocking affinity. As predicted, three residue changes in LCa\textsubscript{1} (N\textsuperscript{3018}, F\textsuperscript{310} and F\textsuperscript{412}) replaced with DHP-sensing residues in respective positions of Ca\textsubscript{1.2}, (Q\textsuperscript{3018}, Y\textsuperscript{3109} and M\textsuperscript{412}) produces snail LCa\textsubscript{1} channel that is not readily separable biophysically or pharmacologically using standard calcium channel blockers to mammalian Ca\textsubscript{1.2} channels.

**Materials and Methods**

**Calcium channels and mutagenesis.** Coding region for the L-type calcium channel, dubbed “LCa\textsubscript{1}” (Genbank: AF484079, 2,078 aa) from the pond snail, *Lymnaea stagnalis*, was previously cloned into bicistronic vector pIRE32-EGFP (Clonetech) and expressed in HEK-293T cells.\textsuperscript{33} Plasmids for rat subunits, Ca\textsubscript{1.2} or \( \alpha\textsubscript{1aC} \) (NM\_012517, 2,170 aa), rat \( \alpha\textsubscript{1D} \) (NM\_012919, 1,091 aa) and rat \( \beta\textsubscript{1B} \) (NM\_017346, 597 aa) were a generous gift from Terry Snutch (Univ. British Columbia) via Gerald Zamponi (Univ. of Calgary). Site directed mutagens (I1506M/F1202Y/N1093Q/N1093A, N1093S) of LCa\textsubscript{1} were created by Quikchange Mutagenesis method (Stratagene) using overlapping PCR primers containing 3.5 kb subclone of LCa\textsubscript{1} channel. All
Channels

Whole cell recording. HEK-293T cells transfected with calcium channel subunits harbored in mammalian expression vectors were heterologously expressed, by calcium-phosphate transfection into human embryonic kidney cell line (HEK293T, M. Calos, Stanford University) at 40–50% confluency. HEK293T cells were cultured in DMEM with 10% FBS and supplemented with 1 mM sodium pyruvate and 0.5% (v/v) penicillin-streptomycin solution. After overnight transfection, the cells were washed three times with culture media and incubated at 28°C in a humidified, 5% CO₂ chamber for seven days. Cells were replated in 60 mm (diameter) sterile Petri dishes containing eight poly-lysine coated glass coverslips (Circles No. 1-0.13 to 0.17 mm thick; Size: 12 mm), (Fisher Scientific Canada) and allowed to recover at 37°C for four hours then left at 28°C for at least three days before patching. Complete details of our methods for optimizing the transfection and recording of ion channels is available as a video journal entry at: http://www.jove.com/index/Details.stp?ID = 2314.

Transient transfection of mammalian cells. Ten μg of calcium channel subunits harbored in mammalian expression vectors were completely sequenced before and after cassette insertion into the full-length LCa1 channel using flanking PstI restriction sites.

**Whole cell recording.** HEK-293T cells transfected with calcium channel subunits were identified by green fluorescence, emitted by EGFP on the bicistronic pIRES2-EGFP plasmid (Clonetech) containing LCa1 calcium channels or by coexpression of pEGFP (Ca1.2). Electrophysiological recordings were carried out in voltage-clamp mode at room temperature, with an Axopatch 200B or Multiclamp 700B amplifier (Axon Instruments, Union City, CA), while monitored by an epifluorescence microscope (Axiovert 40 CFL, Zeiss Canada, Toronto, Ontario). Cells were bathed in external solution containing barium as the charge carrier (15 mM BaCl₂, 1

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**Figure 9.** DHP blocker (S)-isradipine binding residues in Ca1.2. (A) Structure of (S)-isradipine and (S)-Bay K8644. Approximate positions of the channel residues, which contribute to the (S)-isradipine binding site, along with a Ca²⁺ ion chelated by two selectivity-filter glutamates are indicated on (S)-isradipine. Red labels highlight Ca1.2 residues, which are different in LCa1. (B) Model of (S)-isradipine in the calcium binding pocket. S6, P and S5 helices are shown, respectively, by smooth ribbons, sharp ribbons and strands. Repeats I, II, III and IV are cyan, cyan, green and purple, respectively. Ca²⁺ ions are shown as yellow spheres. The model is viewed along P-helix of repeat III, from a channel point most distant from the selectivity filter. The ligand is space-filled. DHP-sensing residues are shown as sticks, except for M₄ᵢ₁₂ and I₄ᵢ₈, which are space-filled and colored magenta.
mM MgCl₂, 10 mM HEPES, 40 mM TEA-Cl, 72.5 mM CsCl, 10 mM Glucose, adjust pH to 7.2 with TEA-OH, filter through 0.22 μm filter). Patch pipettes (2–5 MΩ, World Precision Instruments, Sarasota, Florida) were filled with internal solution (108 mM Cs-methanesulfonate, 4 mM MgCl₂, 9 mM EGTA, 9 mM HEPES, adjust pH to 7.2 with CsOH, filter through 0.22 μm filter). Voltage commands were generated and data were acquired using a PC computer equipped with a Digidata 1440A interface in conjunction with pClamp10.1 software (Molecular Devices, Sunnyvale, California). Recorded currents were filtered at 10 kHz using a low-pass Bessel filter and digitized at a sampling frequency of 2 kHz. Only recordings with minimal leak (<10%) were used for analysis, and offline leak subtraction was carried out using the Clampfit 10.1 software (Molecular Devices, Sunnyvale, California). Series resistance was compensated to 70% (prediction and correction; 10 μs lag). Electrophysiology figures were illustrated in Origin 8. All values are expressed as the mean ± SEM, with statistical analyses using a one-way ANOVA.

**Voltage clamp protocols.** Current-voltage relationships were obtained by holding cells at -100 mV before stepping to test potentials ranging from -50 to +60 mV for 150 ms. Ca²⁺ current activation curves were constructed by converting the peak current values from each current-voltage relationship data set to conductance using the equation

\[
g_{\text{Ca}} = \frac{I_{\text{peak}}}{(V_{\text{command}} - E_{\text{Ca}})}
\]

where \(I_{\text{peak}}\) is the peak current, \(V_{\text{command}}\) is the command pulse potential and \(E_{\text{Ca}}\) the Ca²⁺ reversal potential as determined by linear extrapolation of the current values in the ascending portion of the current-voltage relationships. Conductance values were then normalized and individually fitted with the Boltzmann equation:

\[
g/g_{\text{max}} = \left(1 + \left(\exp(-\frac{V_{\text{command}} - V_{1/2}}{k})\right)\right)^{-1}
\]

where \(g\) is the peak conductance, \(g_{\text{max}}\) is the maximal peak Ca²⁺ conductance, \(V_{\text{command}}\) is the conditioning potential, \(V_{1/2}\) is the half maximal activation and \(k\) is the activation slope factor.

The voltage dependence of inactivation were measured as the fraction of peak currents at a test depolarization step to +10 mV from -100 mV holding potential, after steady-state voltage conditions, prepared with a long 20 to 40 s prepulse holding potentials ranging from -90 to +30 mV. Normalized data were averaged and curve fit with a Boltzmann equation

\[
\frac{I/I_{\text{max}}}{1} = 1 + \exp\left(\frac{(V_{\text{max}} - V_{1/2})}{k}\right)
\]

where \(I\) is the peak test pulse current, \(I_{\text{max}}\) is the peak test pulse current when the conditioning pulse was -100 mV, \(V_{\text{max}}\) and \(V_{1/2}\) are the conditioning potential and the half maximal inactivation, respectively and \(k\) is the inactivation slope factor. Kinetics of activation, inactivation and deactivation were determined by fitting mono-exponential functions over the growing or decaying phases of each current trace using the software Clampfit 10.1.

**Drugs.** Isradipine (I6658; Sigma Aldrich, Oakville, Ontario) was prepared in stock solutions of DMSO. Isradipine serial dilutions were prepared in external recording solution with 0.1% DMSO, and applied by Smartsquirt (Automate Scientific, Berkeley, California) microperfusion -80 μm from recorded cells. Drug effects were measured after current size equilibrated for at least 30 seconds. (S)-(−)-BAYK8644 (B133; Sigma Aldrich) was prepared in a stock solution of DMSO. On the day of experimentation, frozen drug aliquots were reconstituted in external recording solution and 25X stock solutions for each drug amount were prepared by serial dilution. (S)-BAYK was pipetted into the recording dish and a minimum of 20 sweeps passed and the cell current size equilibrated between drug additions.

**Molecular modeling.** AKvAP-based model of Ca₁,2 with 1,4-dihydropyridines was used to visualize the residues mutated in this study and analyze ligand-channel interactions.

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