Supplementary Discussion

**Benzothiazepine Binding in CaVAb**
Diltiazem and other benzothiazepines inhibit CaV1.2 channels in a frequency-dependent manner consistent with pore block. Like the phenylalkylamines, they are primarily used in treatment of cardiac arrhythmias. They bind to a distinct receptor site that is allosterically coupled to the dihydropyridine and phenylalkylamine sites. Mutagenesis studies revealed a set of amino acid residues required for diltiazem binding that overlaps partially with those for phenylalkylamine binding. Diltiazem and other benzothiazepines are larger and more rigid than the phenylalkylamines, and we were unable to observe diltiazem bound in our CaVAb crystals. We assume from these results that its binding is not well accommodated in the conformations of CaVAb that are accessible in our crystallization experiments. Hopefully, ongoing studies will result in capture of bound diltiazem and determination of the structural basis for its Ca\(^{2+}\) antagonist actions.

**Functional Properties and Drug Inhibition of Site-Directed Mutants**
We analyzed the functional properties of the site-directed mutants we used for structure-function analysis carefully by whole-cell voltage clamp (Extended Data Figs. 1, 3, and 6). The I199S mutation had no effect on the voltage dependence of activation of CaVAb or on the kinetics of the early and late phases of inactivation (Extended Fig. 1a, b). However, this mutation shifted the voltage dependence of steady-state inactivation by -9.1 mV (Extended Data Fig. 1d). This enhanced inactivation would have increased drug-binding affinity by itself; therefore, the changes in drug-binding affinity that we report for I199S represent minimum values for the actual effect of the mutation on drug binding *per se*. Drug block of the I199S mutant accumulated during repetitive depolarizations in the presence of amlodipine or nimodipine (Extended Data Fig. 1e, f). It was surprising that the I199S mutation had a stronger effect on inhibition of CaVAb by nimodipine than on inhibition by amlodipine (Figs. 1 and 2; Extended Data Fig. 1). We speculate that the amino group on the aliphatic chain attached to the dihydropyridine ring of amlodipine may allow it to interact more effectively with the substituted Ser residue in the I199S mutant (Fig. 1f).

Although W195 is the native amino acid residue in NaVAb and CaVAb, we used Y195 as the standard construct for our structural work because it yielded a significantly higher fraction of high-resolution structures (see Methods). Substituting W195 caused shift of +9.8 mV in the voltage dependence of activation and made it less steeply voltage dependent, but it had no effect on the kinetics or voltage dependence of the early or late phases of inactivation (Extended Data Fig. 3a-d). We would not expect these small changes in channel function to substantially affect our measurements of drug affinity. Inhibition of the Y195 construct by
nimodipine accumulated during repetitive depolarizations and yielded an IC$_{50}$ of 508 nM at steady state (Extended Data Fig. 3e, f).

The mutation T206S had only a minor effect on the voltage dependence of activation, but it completely prevented the early phase of inactivation during test pulses, such that we could not estimate its effect on the voltage dependence of steady-state inactivation (Extended Data Fig. 6). Inactivation enhances drug binding in general; therefore, we were concerned that the effect of this mutation on inactivation may be dominant in studies of drug block of this mutant. To examine this point, we measured drug block in the resting state and in steady state following a series of depolarizing pulses (see main text and Fig. 4). Because we found substantial effects of this mutation on resting-state block as well as state-dependent block, we can conclude that the mutation of T206 per se reduces drug binding, in addition to any effects of the loss of inactivation.

**Drug Binding Breaks Symmetry**

Although Na$_V$Ab is a homotetramer, we determined its structure using a single site mutant (I217C) in the I222 space group with two neighboring subunits in the asymmetric unit. Strictly speaking, the final structure of the nearly four-fold symmetric Na$_V$Ab channel has two-fold symmetry and a dimer-of-dimers structure. We subsequently determined the structure of WT Na$_V$Ab in two inactivated states, which were crystallized in the P42 space group and have a more substantial breakdown of four-fold symmetry. In these inactivated states, two S6 segments move toward the central axis and two move away to produce a parallelogram arrangement of elements at the ion selectivity filter, central cavity, and intracellular activation gate. The inactivated channel nevertheless has two-fold symmetry relating its halves. Here we find that drug binding induces a breakdown of this two-fold symmetry and makes the channel completely asymmetric. The drug-bound structures were determined and best refined in the P21221 space group with all four subunits in the asymmetric unit. Binding of a single dihydropyridine induces a substantial change in quaternary structure, as illustrated in Fig. 3. This conformational change subtly alters the shape of the dihydropyridine-binding sites in the three unoccupied subunits (Extended Data Fig. 8), making the two neighboring sites too wide at the bottom and the diagonal site too narrow at the top. These structural changes could reduce drug access and drug-binding affinity in the three unoccupied subunits, resulting in the singly occupied state that we have captured in our crystals.

In our Ca$_V$Ab structures with Br-verapamil bound (Fig. 4), drug binding also induces a complete breakdown of the two-fold symmetry we have previously observed with Na$_V$Ab. Once again, the structures were defined in P21221 space group with all four subunits in the asymmetric unit. Non-crystallographic symmetry restraints were not used in the refinement because the spatial relationships between the alpha helices in the voltage-sensing modules and the pore module were all distinct among the four subunits.
(Extended Data Fig. 9a), and the C-terminal halves of the S6 helices were tilted relative to the rest of the pore module. The bound drug molecule stretches between two adjacent subunits, making contacts with Thr206 in both subunits plus adjacent amino acid residues that differ between the two subunits. Remarkably, the bound drug brings two of the subunits closer to each other, compared to the other pairs of subunits (Extended Data Fig. 9b). The size of Br-verapamil makes binding of two molecules in the central cavity in symmetrically oriented positions unlikely. Moreover, it would not be possible for a second drug molecule to close the distance between the two subunits to which it is bound in the same way that we observe for the single drug-bound complex (Extended Data Fig. 9b). Together, these features make the drug-bound complex completely asymmetric.

The conformational changes induced by binding of dihydropyridines and Br-VERPamil are different from each other, and they are likely to contribute to the complex allosteric interactions between the bound drugs. Interplay between these drug-induced conformational changes and the voltage-dependent conformational changes induced in the voltage-sensing module by changes in membrane potential are likely to contribute to the voltage-dependent drug binding that is a hallmark of the complex actions of these drugs on Cav1.2 channels in cardiac and vascular smooth muscle myocytes. These multifaceted structural changes contribute to the differences in the voltage dependence of action of these drugs and to their different uses in treatment of hypertension, angina pectoris, and cardiac arrhythmia.

Direct Binding of Ca\(^{2+}\) to Carboxylate Groups in the Ion Selectivity Filter

As a consequence of the breakdown of symmetry in CavAb with a dihydropyridine bound, the Ca\(^{2+}\) ion bound in Site 1 in the ion selectivity filter moves from the central axis toward the subunit to which the drug is bound (Fig. 3; Extended Data Fig. 5a-d). We interpret our structure to indicate that Ca\(^{2+}\) binds directly to the carboxylate of D178 when a dihydropyridine binds to CavAb. This conclusion is based on two considerations. First, the distance of 2.8-3.2 Å is too small for a water molecule to bridge the Ca\(^{2+}\) and carboxylate. Second, the distances observed for direct interaction of Ca\(^{2+}\) with carboxylates are 2.2-2.6 Å in SERCA ATPase and 2.4-2.9 Å in a sodium-calcium exchanger\(^{10,11}\). As these specialized sites are designed to bind Ca\(^{2+}\) with very high affinity as part of their physiological function in ion transport, we think that these results are compatible with direct binding of Ca\(^{2+}\) to the carboxylates of D178 at a distance of 2.8-3.2 Å induced by drug binding in CavAb.

Efficacy and Safety of Ca\(^{2+}\) Antagonist Drugs

Ca\(^{2+}\) antagonist drugs are relatively safe in therapeutic use (eg., amlodipine)\(^{12}\), especially when compared to the Na\(^{+}\) channel blockers used for treatment of cardiac arrhythmias. However, toxic side effects are well-
known and can be life-threatening\textsuperscript{13}. Toxicity of phenylalkylamines arises primarily from depression of ventricular contractility, which is caused by tonic inhibition of \textit{Ca}\textsubscript{V}1.2 channels in ventricular myocytes\textsuperscript{13}. Structure-based drug design could enhance both efficacy and safety of these drugs through development of novel agents that would have greater frequency dependence of action, thereby further directing drug binding to the cardiac myocytes that are firing action potentials at an inappropriately rapid rate. Toxicity of dihydropyridines arises from both depressed ventricular contractility and depressed vascular smooth muscle contractility\textsuperscript{13}. Structure-based drug design could contribute to mitigation of this toxicity. Stronger voltage dependence of binding would direct drug binding further toward blocking \textit{Ca}\textsubscript{V}1.2 channels in vascular smooth muscle, allowing lower effective doses with less effect on ventricular function. Toxic overdoses of both phenylalkylamines and dihydropyridines cause cardiac arrhythmias\textsuperscript{14,15}. Fine-tuning drug structure could potentially prevent off-target pore block of sodium channels by these drugs, as illustrated at the atomic level for a dihydropyridine in Fig. 4g-i. Off-target sodium channel block by \textit{Ca}\textsuperscript{2+} antagonist drugs could contribute to generation of lethal arrhythmias, just like excess sodium channel block by antiarrhythmic drugs at high doses. We hope that the structural studies presented here mark a first step toward future advances in efficacy and safety of therapy for cardiovascular diseases.
