Structural basis for Ca\(^{2+}\) selectivity of a voltage-gated calcium channel

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Voltage-gated calcium (CaV) channels catalyse rapid, highly selective influx of Ca\(^{2+}\) into cells despite a 70-fold higher extracellular concentration of Na\(^+\). How CaV channels solve this fundamental biophysical problem remains unclear. Here we report physiological and crystallographic analyses of a calcium selectivity filter constructed in the homotetrameric bacterial NaV channel NaV\(_\text{Ab}\). Our results reveal interactions of hydrated Ca\(^{2+}\) with two high-affinity Ca\(^{2+}\)-binding sites followed by a third lower-affinity site that would coordinate Ca\(^{2+}\) as it moves inward. At the selectivity filter entry, Site 1 is formed by four carboxyl side chains, which have a critical role in determining Ca\(^{2+}\) selectivity. Four carboxyls plus four backbone carbonyls form Site 2, which is targeted by the blocking cations Cd\(^{2+}\) and Mn\(^{2+}\), with single occupancy. The lower-affinity Site 3 is formed by four backbone carbonyls alone, which mediate exit into the central cavity. This pore architecture suggests a conduction pathway involving transitions between two main states with one or two hydrated Ca\(^{2+}\) ions bound in the selectivity filter and supports a ‘knock-off’ mechanism of ion permeation through a stepwise-binding process. The multi-ion selectivity filter of our CaV\(_\text{Ab}\) model establishes a structural framework for understanding the mechanisms of ion selectivity and conductance by vertebrate CaV channels.

Ca\(^{2+}\) ions flow through CaV channels at a rate of \(-10^6\) ions s\(^{-1}\), yet Na\(^+\) conductance is \(-500\)-fold lower\(^1\). Such high-fidelity, high-throughput CaV channel performance is important in regulating intracellular processes such as contraction, secretion, neurotransmission and gene expression in many different cell types\(^2\). Because the extracellular concentration of Na\(^+\) is 70-fold higher than Ca\(^{2+}\), these essential biological functions require CaV channels to be highly selective for Ca\(^{2+}\) in preference to Na\(^+\), even though Ca\(^{2+}\) and Na\(^+\) have nearly identical diameters (\(-\sim 2\) Å). Ion selectivity of CaV channels is proposed to result from high-affinity binding of Ca\(^{2+}\), which prevents Na\(^+\) permeation. Fast Ca\(^{2+}\) flux through CaV channels is thought to use a ‘knock-off’ mechanism in which electrostatic repulsion between Ca\(^{2+}\) ions within the selectivity filter overcomes tight binding of a single Ca\(^{2+}\) ion\(^3\)\(^-\)\(^8\). Most of these mechanisms require a multi-ion pore, yet extensive mutational analyses of ion selectivity and cation block of vertebrate CaV channels support a single high-affinity Ca\(^{2+}\)-binding site\(^9\)\(^-\)\(^14\).

CaV channels contain a single ion-selective pore in the centre of four homologous domains\(^2\). The central pore is lined by the transmembrane segments (S) S5 and S6 and the intervening ‘Pore (P)-loop’ from each domain in a four-fold pseudosymmetrical arrangement. The four voltage-sensing modules composed of S1–S4 transmembrane helices are symmetrically arranged around the central pore. CaV channels are members of the voltage-gated ion channel superfamily and are closely related to voltage-gated Na\(^+\) (NaV) channels. Three structures of homotetrameric bacterial NaV channels open the way to elucidating the structural basis for ion selectivity and conductance of vertebrate NaV and CaV channels\(^1\)\(^-\)\(^15\)\(^-\)\(^17\), which probably evolved from the bacterial NaChBac family and retained similar structures and functions (Supplementary Fig. 1)\(^18\)\(^-\)\(^20\). Interestingly, mutation of three amino-acid residues in the selectivity filter of NaChBac is sufficient to confer Ca\(^{2+}\)-selectivity\(^2\)\(^1\). We introduced analogous mutations into the bacterial NaV channel NaV\(_\text{Ab}\) to create CaV\(_\text{Ab}\) and carried out electrophysiological and X-ray crystallographic analyses to determine the relative permeability of Ca\(^{2+}\) and define ion-binding sites in the selectivity filter. Our systematic analyses of CaV\(_\text{Ab}\) and intermediate derivatives provide structural and mechanistic insights into Ca\(^{2+}\) binding and ion permeation and suggest a conductance mechanism involving two energetically similar ion-occupancy states with one or two hydrated Ca\(^{2+}\) ions bound.

**Structure and function of CaV\(_\text{Ab}\)**

NaV\(_\text{Ab}\) channels have four identical pore motifs (\(175\)TLEWSM\(_{181}\)) that form the ion selectivity filter\(^15\). The side chains of E177 form a high-field-strength site (Site\(_\text{E177}\)) at the outer end of the filter, whereas two additional potential Na\(^+\)–coodination sites, a central site (Site\(_\text{C181}\)) and an inner site (Site\(_\text{C178}\)), are formed by the backbone carbonyls of L176 and T175 (ref. 15). To create CaV\(_\text{Ab}\), E177, S178 and M181 were substituted with Asp, resulting in a mutant with the pore motif \(175\)TLDWSD\(_{181}\) (underlined letters indicate mutated residues). CaV\(_\text{Ab}\) was expressed in *Trichoplusia ni* cells (High5) and analysed by whole-cell voltage clamp to determine its ion selectivity. In contrast to NaV\(_\text{Ab}\), which does not conduct extracellular Ca\(^{2+}\) ions but carries outward Na\(^+\) current (Fig. 1a, b), CaV\(_\text{Ab}\) conducts inward Ca\(^{2+}\) current in a voltage-dependent manner (Fig. 1c, d). Complete titration curves for Ca\(^{2+}\) in the presence of Ba\(^{2+}\) as the balancing divalent cation (see Methods) revealed inhibition of Ba\(^{2+}\) current by low concentrations of Ca\(^{2+}\) followed by increases in Ca\(^{2+}\) current at higher Ca\(^{2+}\) concentrations (Fig. 1e). These results demonstrate the anomalous mole fraction effect characteristic of vertebrate CaV channels. Comparable experiments with Na\(^+\) as the balancing cation were not possible because of the instability of the High5 cells in solutions with low divalent cation concentrations. The reversal potential for Ca\(^{2+}\) current under bi-ionic conditions closely follows the expectation for a highly Ca\(^{2+}\)-selective conductance (30.6 ± 2.3 mV decade\(^{-1}\), Fig. 1f and Supplementary Fig. 2), and CaV\(_\text{Ab}\) selects Ca\(^{2+}\) 382-fold over Na\(^+\) under our standard conditions.

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The three negative potentials at the outer entry to the selectivity filter is more negative observed for NaChBac1. The depolarizations.

The 3.2 Å resolution structure of the mutant 175TLDDWSD181 (Fig. 1a), and it still favours Ca2+ due in place of the final Asp, as observed in one domain of mammalian CaV channels (Supplementary Fig. 1), and it still favours Ca2+ over Na+ by more than 100-fold (Fig. 1g).

We crystallized and determined the structure of CaVAb and its derivatives by molecular replacement using the NaVAb structure (PDB code 3RVY) as the search template (Supplementary Table 1). The overall structure of CaVAb is very similar to that of NaVAb, with a root mean squared deviation (r.m.s.d.) of 0.4 Å (Fig. 1h). However, the electrostatic potential at the outer entry to the selectivity filter.

The Ca2+–binding sites in the permeation pathway

The 3.2 Å resolution structure of the mutant 175TLDDWSD181 in the presence of 10 mM Ca2+ reveals electron densities in the selectivity filter consistent with three Ca2+ ions aligned on the central axis (Fig. 2a). In the outer vestibule leading to the selectivity filter, there are two additional less-intense on-axis peaks associated with weaker surrounding densities. To confirm the identity of the bound ions, we collected X-ray diffraction data at a wavelength of 1.75 Å and calculated the $F_{\text{Ca}^2+} - F_{\text{Na}^+}$ anomalous difference map. Two strong peaks followed by a weaker peak on the intracellular side were found in the selectivity filter along the ion-conduction pathway, verifying three binding sites for Ca2+ (Fig. 2b).

The Ca2+ ion at Site 1 is predominantly coordinated by the carboxyl groups of D178 (Site$_{\text{HFS}}$ in NaVAb), which define a plane at the selectivity filter entrance on the extracellular side of the bound Ca2+ ion (Fig. 2b). The distance between the carboxyl oxygen and Ca2+ is about 4.0 Å. This distance suggests that the ion binds at this site in a hydrated form because the ionic diameter of Ca2+ is 2.28 Å, too small to interact with the carboxylic anions directly but appropriate for interaction through bound water molecules. Further into the pore, the four acidic side chains of D177 (Site$_{\text{CEN}}$ in NaVAb) are located along the wall of the selectivity filter rather than projecting into the lumen, thereby also allowing the binding of a fully hydrated Ca2+ ion (Fig. 2b). Different from Site 1, this central Ca2+–binding site (Site 2) is surrounded by a box of four carboxylate oxygen atoms from D177 above and four backbone carbonyl oxygen atoms from L176 below (Site$_{\text{CEN}}$ in NaVAb), with oxygen–Ca2+ distances of 4.5 Å and 4.2 Å, respectively (Fig. 2b). At the intracellular side of the pore, the third Ca2+–binding site (Site 3) is composed of one plane of four carboxyls from T175 (Site$_{\text{CEN}}$ in NaVAb), which point inward to the lumen (Fig. 2b).

Here the Ca2+ ion lies nearly on the same plane as T175 carboxyls. The chemical environment of Site 3 hints at a lower affinity, consistent with its role in exit of Ca2+ from the selectivity filter into the central cavity. Throughout the selectivity filter, the oxygen–Ca2+ coordination distances are in the range of 4.0–5.0 Å, suggesting that the bound Ca2+ ion is continuously stabilized in a fully hydrated state when it passes through the pore. We observed diffuse electron density and in favourable cases
at its mouth by recognizing the Ca$^{2+}$-$\text{H}_2\text{O}$ hydration complex and conduct Ca$^{2+}$ by fitting the Ca$^{2+}$-$\text{H}_2\text{O}$ hydration complex into the pore. Because Ca$^{2+}$ is more electropositive than Na$^+$, it should bind more tightly in the ion selectivity filter of Ca$_{IV}$Ab, providing a mechanistic basis for the block of Na$^+$ permeation by Ca$^{2+}$ at low Ca$^{2+}$ concentration and preferential permeation of Ca$^{2+}$ at higher Ca$^{2+}$ concentration (see Discussion).

**Functional roles of key selectivity filter residues**

Measurements of bi-ionic reversal potentials revealed that the relative permeability of different Ca$_{IV}$Ab intermediate constructs for Ca$^{2+}$ follows the order of CavAb (175TLDDLWSM$^{181}$) > 175TLDDLWSN$^{181}$ > 175TLDDLWSD$^{181}$ > 175TLDDLWSM$^{181}$ > Na$_v$Ab (175TLEWSM$^{181}$) > 175TLDDLWSM$^{181}$ (Fig. 1g and Supplementary Fig. 2). A comparison of the Ca$^{2+}$ selectivity ratios between 175TLDDLWSM$^{181}$ and 175TLEWSM$^{181}$ (Na$_v$Ab) shows that substitution of S178 with Asp is sufficient to convert the selectivity from Na$^+$ to Ca$^{2+}$ with >100-fold change in $P_{\text{Ca}/P_{\text{Na}}}$ (Fig. 1g). Placement of the Asp carboxyl side chain at this position allows for the formation of the first hydrated Ca$^{2+}$-binding site in the selectivity filter (Fig. 2c and Supplementary Fig. 6). By contrast, S178 in Na$_v$Ab binds Ca$^{2+}$ directly by displacing its hydration shell, which blocks conductance of both Na$^+$ and Ca$^{2+}$ (Fig. 2d). Therefore, formation of Site 1 for binding hydrated Ca$^{2+}$ is both necessary and sufficient for conferring Ca$^{2+}$ selectivity over Na$^+$ to Na$_v$Ab. The Ca$^{2+}$ selectivity ratio of Ca$_{IV}$Ab (175TLDDLWSM$^{181}$) is 5.5-fold higher than 175TLDDLWSM$^{181}$ (Fig. 1g). This functional difference reflects a role of Site 2 in adjusting Ca$^{2+}$ selectivity. Different from the side chains of D177 in Ca$_{IV}$Ab (175TLDDLWSM$^{181}$), which interact with the Ca$^{2+}$ ion (Fig. 2e), the carboxyl group of E177 in 175TLDDLWSM$^{181}$ swings away from the selectivity filter and forms a hydrogen bond with D181 and the main-chain nitrogen atoms of S180 (Fig. 2f and Supplementary Fig. 7). Site 2 in 175TLDDLWSM$^{181}$, therefore, is exclusively formed by the four carboxyl oxygen atoms of L176, which conceivably leads to a lower Ca$^{2+}$-binding affinity and a decreased Ca$^{2+}$ selectivity. This comparison highlights both the importance of Site 2 in supporting high Ca$^{2+}$ selectivity and the critical role of the backbone carbonyl groups of L176 in constructing this ion-binding site.

Distinct from D177 and D178, the N181 residue of 175TLDDLWSM$^{181}$ lies outside of the ion-conducting pore and is not directly involved in Ca$^{2+}$ ion coordination. In close proximity to the carboxyl groups of D178, which form a ring that lines the perimeter of the pore entryway, the side chain of N181 embraces the perimeter of the D178 ring by donating a hydrogen bond to its side-chain carboxyls (Fig. 3a). Such a structural arrangement is also found in Ca$_{IV}$Ab (175TLDDLWSM$^{181}$) (Fig. 3b), although the more electronegative environment created by the extra negatively charged residue, D181, probably attracts Ca$^{2+}$ more strongly and confers a 4- to 5-fold higher degree of Ca$^{2+}$ selectivity to Ca$_{IV}$Ab (175TLDDLWSD$^{181}$) in comparison to 175TLDDLWSM$^{181}$ (Fig. 1g and Supplementary Fig. 3).

175TLDDLWSM$^{181}$, which has the hydrophobic residue M181 packed next to the D178 ring, is the only Ca$_{IV}$Ab intermediate that does not conduct Ca$^{2+}$ (Supplementary Fig. 2). The crystal structure of this mutant reveals a blocking Ca$^{2+}$ ion tightly bound at Site 1 in a dehydrated state with an oxygen–ion distance of 2.3 Å (Fig. 3c). Superposition analysis shows few structural differences between 175TLDDL WS$^{181}$ and 175TLDDLWS$^{181}$, except for the side chain of D178, which is fixed by N181 in 175TLDDLWSN$^{181}$ but unconstrained in 175TLDDLWS$^{181}$ (Fig. 3a, c). This comparison indicates that N181 in 175TLDDLWS$^{181}$ and D181 in Ca$_{IV}$Ab have critical roles in engaging D178 and allowing the reversible binding of the Ca$^{2+}$-$\text{H}_2\text{O}$ hydration complex for active Ca$^{2+}$ conductance. Although the subtle difference in Ca$^{2+}$ selectivity between 175TLDDLWS$^{181}$ and 175TLDDLWSD$^{181}$ seems to argue against this conclusion (Fig. 1g), E177 in 175TLDDLWS$^{181}$ actually has a structural role equivalent to that of N181 in 175TLDDLWSM$^{181}$ — by pointing away from the selectivity filter lumen, E177 forms a carbonate–carboxylate

discrete water molecules surrounding the bound Ca$^{2+}$, consistent with the presence of an inner shell of bound waters of hydration (Supplementary Fig. 5).

Although the anomalous difference map did not resolve clear peaks at the outer vestibule beyond the selectivity filter, we interpret the two on-axis $2F_o - F_c$ densities above the three Ca$^{2+}$ sites as two additional Ca$^{2+}$ ions poised to enter the pore (Fig. 2a). This assignment is supported by the surrounding eight islets of density, which probably represent eight stabilized water molecules. Just as at Site 2 in the selectivity filter, these eight water molecules appear to serve as a square antiprism cage coordinating a hydrated Ca$^{2+}$ ion at the centre (Fig. 2a). The second Ca$^{2+}$ ion located at the bottom of this cage is ~4.5 Å away from the four carboxyl oxygen atoms of D178, suggesting that part of its second hydration shell is replaced by D178 before the ion enters the selectivity filter. The selectivity filter, therefore, appears to select Ca$^{2+}$...
Figure 3 | Ion binding and block of CaVAb and its derivatives. **a, b**, Top view of Site 1 with a hydrated Ca\(^{2+}\) ion coordinated by D178 with the help of N181 and D181 in \(^{175}\)TLDDWSN\(_{181}\) and \(^{175}\)TLDDWSN\(_{181}\) (CaVAb), respectively. **c**, Binding of a dehydrated Ca\(^{2+}\) ion at Site 1 in the nonconductive \(^{175}\)TLDDWSM\(_{181}\) mutant. **d**, Coordination of a hydrated Ca\(^{2+}\) ion at Site 1 of the \(^{175}\)TLDDWSM\(_{181}\) mutant. Despite the absence of a polar residue at amino acid 181, E177 in \(^{175}\)TLDDWSM\(_{181}\) is able to hold D178 in place to allow the binding of a hydrated Ca\(^{2+}\) ion. **e, f**, Block of Ca\(^{2+}\) conductance by the pair with D178 and holds it in a conduction-competent position (Fig. 3d and Supplementary Fig. 8).

**Block of Na\(_V\)Ab and Ca\(_V\)Ab channels by divalent cations**

Cd\(^{2+}\), Mn\(^{2+}\) and other inorganic cations are effective blockers of Ca\(_V\) channels\(^1\). Block of Ca\(^{2+}\) conductance of Ca\(_V\)Ab by Cd\(^{2+}\) and Mn\(^{2+}\) gives \(K_b\) values of 1.78 \(\mu\)M for Cd\(^{2+}\) and 526 \(\mu\)M for Mn\(^{2+}\) (Fig. 3e, f, blue). Cd\(^{2+}\) has a lower affinity and Mn\(^{2+}\) has a higher affinity for block of \(^{175}\)TLDDWSN\(_{181}\) (Fig. 3e, f, red). Crystals with bound Cd\(^{2+}\) and Mn\(^{2+}\) were obtained by soaking CaVAb crystals in a cryo-solution containing these heavy metal ions, and the anomalous difference map was calculated from a data set collected at 1.75 Å wavelength. The structures show that both Cd\(^{2+}\) and Mn\(^{2+}\) bind in the selectivity filter at the central site (Site 2), which is coordinated by the side chains of the four D177 residues and the carbonyl groups of L176 (Fig. 3g, h). Locked at this site, these blocking ions would inhibit the Ca\(^{2+}\) current by competitively binding to the high-affinity site required for Ca\(^{2+}\) permeation.

Another important common feature of the two blocking complexes of CaVAb is the block of permeation by binding of a single divalent cation within the selectivity filter, which supports the hypothesis that at least two divalent-cation-binding sites must be located close enough to induce repulsive interactions and allow divalent cation conductance by a knock-off mechanism. Because they are smaller than Ca\(^{2+}\), the bound Cd\(^{2+}\) (\(d = 2.18\) Å) and Mn\(^{2+}\) (\(d = 1.94\) Å) must interact with the selectivity filter through bound waters of hydration, and electron density consistent with bound waters of hydration is observed in our structures (Supplementary Fig. 5).

**Ion binding at the Ca\(^{2+}\) selectivity filter**

To assess the properties of the three Ca\(^{2+}\)-binding sites in the selectivity filter of \(^{175}\)TLDDWSN\(_{181}\), we titrated the concentration of Ca\(^{2+}\) in the cryo-solution and calculated the anomalous difference maps. At low Ca\(^{2+}\) concentration, two strong peaks of approximately equal intensity are found at Site 1 and Site 2 (Supplementary Fig. 9). As the Ca\(^{2+}\) concentration is raised, the electron density of Site 2 is substantially enhanced, but the peak intensity is reduced at Site 1 and remains low at Site 3 (Supplementary Fig. 9). These results suggest that the central site has the highest affinity, whereas Site 3 is the weakest. It is probable that this titration pattern reflects independent binding of Ca\(^{2+}\) to Sites 1, 2 and 3 located in different individual molecules of CaVAb at low Ca\(^{2+}\) concentration, whereas increasing concentrations of Ca\(^{2+}\) saturate Site 2 in most or all individual CaVAb molecules and reduce or eliminate binding at Sites 1 and 3 by repulsion. Importantly, the two flaving sites have lower affinity than the central site, as proposed in the ‘stepwise binding model’ of Ca\(_V\) channel permeation\(^4\). In this model, the presence of flanking sites of intermediate affinity facilitates the movement of Ca\(^{2+}\) into and out of a central high-affinity site, which can result in high ion conductance, even in the limiting case where there is no repulsion between bound ions.

Consistent with high binding affinity, Ca\(^{2+}\) binds at Site 2 with its first hydration shell waters coordinated with eight oxygen atoms from the channel (Fig. 2b and Supplementary Fig. 5). By contrast, Ca\(^{2+}\) at site 1 is mainly stabilized by one plane of four carboxyl groups from D178. The distance between the Ca\(^{2+}\) ion at Site 1 and the carboxyl group of D178 at Site 2 is about 5.5–6 Å. As the Ca\(^{2+}\) ion moves inward, this distance will be reduced enough for D177 to form a stable coordination with the moving Ca\(^{2+}\) ion. This spatial configuration suggests that the two sites are separated by a low energy barrier. The differences of negative charge between D178 and the carbonyls of T175 and the differences in the geometry of their interactions with Ca\(^{2+}\) provide a plausible explanation for the higher Ca\(^{2+}\)-binding affinity at Site 1 than Site 3.

**Ion permeation mechanism**

The three Ca\(^{2+}\)-binding sites in the selectivity filter of \(^{175}\)TLDDWSN\(_{181}\) are separated by a distance of about 4.5 Å, which would result in substantial electrostatic repulsive interactions between bound ions. As in the case of the KcsA channel\(^2\), it is energetically unfavourable for Ca\(^{2+}\) ions to occupy adjacent sites simultaneously. This leads directly to our hypothesis of two interchangeable functional states of the selectivity filter in the crystal structure (Fig. 4a, b). In State 1, Ca\(^{2+}\) ions occupy Site 1 and Site 3. In State 2, a single Ca\(^{2+}\) ion occupies Site 2. These two states might be further coupled with one of the two Ca\(^{2+}\) ions at the outer vestibule ready to enter the pore (Fig. 4c). The transition between these two states occurs either when Ca\(^{2+}\) jumps from Site 1 or Site 3 to Site 2 or a third ion enters on one side of the filter, causing an ion to move into Site 2. It is probable that our crystal structures reflect a mixed population of CaVAb molecules in which only Site 2 is occupied by Ca\(^{2+}\) plus CaVAb molecules in which Site 1 and/or Site 3 are occupied. Because of

\[ \text{IC}_{50} (\text{Cd}^{2+}) = 1.7 \pm 0.04 \mu\text{M}; \text{IC}_{50} (\text{Mn}^{2+}) = 5.9 \pm 0.4 \mu\text{M}; \text{IC}_{50} (\text{Cd}^{2+}) = 388 \pm 7 \mu\text{M}. \]

\[ \text{IC}_{50} (\text{Cd}^{2+}) = 1.78 \pm 0.04 \mu\text{M}; \text{IC}_{50} (\text{Mn}^{2+}) = 5.9 \pm 0.4 \mu\text{M}. \]
select their permeant ions through direct interaction of the dehydrated ions with backbone carbonyls.

Our results reveal an unexpected structural basis for Ca\(^{2+}\) selectivity and conductance in Ca\(_{\text{V}}\)Ab channels, in which most or all interactions of Ca\(^{2+}\) with the pore are made through its inner shell of waters of hydration. A set of three Ca\(^{2+}\) binding sites cooperate in a knock-off mechanism in which the selectivity filter oscillates primarily between two states with either one hydrated Ca\(^{2+}\) bound at the central site or two hydrated Ca\(^{2+}\) ions bound at the distal sites. The high-affinity binding of Ca\(^{2+}\) to Sites 1 and 2 ensures that Na\(^{+}\) and other monovalent cations cannot permeate, while the high Ca\(^{2+}\) concentration in the extracellular solution enables unidirectional flux by driving rapid occupancy of Site 1. The ionic repulsion between Ca\(^{2+}\) ions bound at these sites and their stepwise change in binding affinity work together to allow rapid conductance in spite of the intrinsic high affinity for Ca\(^{2+}\) binding. Although our resolution does not allow us to see all of the waters of hydration that are implied by our structure, we do observe electron density surrounding bound Ca\(^{2+}\) ions at Sites 1, 2 and 3 that we believe represents the inner shell of waters of hydration (Supplementary Figure 5). This electron density is blurred, as if there is a diversity of arrangements of the bound water molecules in individual Ca\(_{\text{V}}\)Ab molecules in our crystals because their hydrogen-bonding requirements can be accommodated in multiple ways between the bound cations and their coordinating carbonyl and carbonyl oxygens that comprise Sites 1, 2 and 3. In our most favourable structure (Supplementary Fig. 5g,h), four discrete water molecules are observed at Site 3. Altogether, we believe that these images provide direct support for the conclusion that bound Ca\(^{2+}\) ions are surrounded by an inner shell of waters of hydration that are dynamic and can easily exchange local hydrogen-binding partners. This is a unique ion conduction mechanism, which allows high-affinity interaction of hydrated Ca\(^{2+}\) ions while mediating their rapid movement from the extracellular vestibule, through the three ion coordination sites of the selectivity filter, through the central cavity, and finally into the cytosol.

Biophysical modelling of Ca\(^{2+}\) permeation in vertebrate Ca\(_{\text{V}}\) channels has led to multiple proposed mechanisms, most of which involve two or more Ca\(^{2+}\)-binding sites, yet only a single high-affinity site that is required for both permeation and Ca\(^{2+}\) block was identified by mutagenesis and physiological analyses. Our results with Ca\(_{\text{V}}\)Ab channels resolve this apparent discrepancy by showing that multiple Ca\(^{2+}\)-binding sites are necessary for permeation, but only Site 2 binds divalent cations with sufficient affinity for block. Ca\(^{2+}\) is conducted as a hydrated cation (Supplementary Fig. 5), consistent with the large estimated functional diameter of vertebrate Ca\(_{\text{V}}\) channels of 6 Å (ref. 26). Detailed structure–function studies of vertebrate Ca\(_{\text{V}}\) channels show that mutations of the four residues equivalent to E177 have distinct effects on Ca\(^{2+}\) conductance and block, implying that domain-specific interactions with Ca\(^{2+}\) have evolved in vertebrate four-domain Ca\(_{\text{V}}\) channels\(^{10,11,27-29}\). Vertebrate Ca\(_{\text{V}}\) channels might share similar molecular mechanisms for Ca\(^{2+}\) permeation and selectivity despite their pseudosymmetrical four-domain configuration.

**Note added in proof:** Crystal structures of isolated pore domains of other bacterial Na\(_{\text{V}}\) channels reveal an open pore conformation for Na\(_{\text{V}}\)Ms (ref. 30) and a binding site for blocking Ca\(^{2+}\) ions in Na\(_{\text{V}}\)Ac (ref. 31), which is formed primarily by the equivalent of Ser 178 in Na\(_{\text{V}}\)Ab.

**METHODS SUMMARY**

Ca\(_{\text{V}}\)Ab and its derivative constructs were expressed in Trichoplusia ni insect cells and purified using anti-Flag resin and size-exclusion chromatography, reconstituted into DMPC:CHAPSO bicelles, and crystallized over an ammonium sulphate solution containing 0.1 M Na-citrate, pH 4.75. The anomalous data sets were collected at 1.75 Å wavelength with crystals soaked in a stabilizing solution containing various concentrations of cation ions. Electrophysiological experiments were performed in T. ni cells using standard protocols.

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**Figure 4 | Catalytic cycle for Ca\(^{2+}\) conductance by Ca\(_{\text{V}}\)Ab.** a, An ionic occupancy state diagram of Ca\(_{\text{V}}\)Ab showing two proposed low energy states and the potential transitions that connect them. Each state of the selectivity filter is represented by a three-box rectangle with Sites 1–3 going from left to right. Green circles represent Ca\(^{2+}\) ions. Note that transitions in the inner circle potentially lead to ion repulsion, which might facilitate conduction. These transitions in the inner circle are more probable than those in the outer circle, as denoted by the bold arrows. b, The structural basis of the ionic occupancy states depicted in the inner circle of the state diagram shown on the left. The clockwise cycle represents a path for inward flux of Ca\(^{2+}\) ions through the selectivity filter. c, Coupling of extracellular Ca\(^{2+}\)-binding sites and the three sites within the selectivity filter in the two proposed ionic occupancy states. When two Ca\(^{2+}\) ions bind to position 1 and 3 in the filter, the entryway Ca\(^{2+}\) ion is placed furthest from the pore (left). When one Ca\(^{2+}\) ions bind to position 2 within the filter, the ion outside the filter is pulled closer to the pore (right).
Online Content Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions L.T., T.M.G.E.-D., J.P., T.S., N.Z. and W.A.C. designed the experiments. J.P. initiated the experimental work. L.T. conducted the protein purification, crystallization and diffraction experiments. T.M.G.E.-D. and T.S. performed physiological studies of Ca2+Ab and related constructs. G.Q.M. and T.M.H. made the constructs and performed the preliminary data collection. All authors interpreted the structures in light of the physiological data. L.T., N.Z. and W.A.C. wrote the manuscript with input from all co-authors. W.A.C. and N.Z. are co-senior authors.

Author Information Coordinates and structure factors have been deposited in the Protein Data Bank under accession codes: 4MS2 (TLDDW5N, 15 mM Ca2+), 4MTF (TLDDW5N, 0.5 mM Ca2+), 4MTG (TLDDW5N, 2.5 mM Ca2+), 4MTO (TLDDW5N, 5 mM Ca2+), 4MVM (TLDDW5N, 10 mM Ca2+), 4MVO (TLDDW5N, 15 mM Ca2+), 4MVQ (TLDDW5N, 15 mM Ca2+), 4MVR (TLDDW5N, 100 mM Mn2+), 4MVS (TLDDW5N, 100 mM Co2+), 4MVZ (TLDDW5N, 15 mM Ca2+), 4MVF (TLDDW5N, 15 mM Ca2+), 4MVW (NawAb, 15 mM Ca2+). Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to N.Z. (nzheng@uw.edu) or W.A.C. (wcatt@uw.edu).
METHODS

Protein expression and purification. The pFastBac-Flag-NaVAb(II17C) that was used as the genetic background for CaVAb constructs was described previously. CaVAb and its derivatives were reconstituted into DPPC:CHAPSO (Anatrace) bilayers according to standard protocols. The protein–bicelle preparation and a well solution containing 1.8–2.0 M ammonium sulphate, 100 mM Na-citrate, pH 5.0, was mixed with a 1:1 ratio and set up in a hanging-drop vapour-diffusion format. The Ca2+-derivative crystals were obtained by soaking CaVAb and other mutant crystals in a cryo-protection solution (0.1 M Na-acetate, pH 5.0, 26% glucose and 2.0 M ammonium sulphate) containing the indicated concentrations of Ca2+ for 40–60 min at 4°C. The Cd2+ and Mn2+ derivatives were obtained by soaking CaVAb in the presence of 100 mM Cd2+ or Mn2+, respectively. Crystals were then plunged into liquid nitrogen and maintained at 100 K during all data collection procedures.

All anomalous diffraction data sets were collected at 1.75 Å with the same synchrotron radiation source (Advanced Light Source, BL8.2.1). To optimize the anomalous signal, the data sets were collected using the ‘inverse beam strategy’ with the wedge size of 5°.

Structure determination, refinement and analyses. X-ray diffraction data were integrated and scaled with the HKL2000 package and further processed with the CCP4 package. The structure of CaVAb and its derivatives were solved by molecular replacement by using an individual subunit of the NaVAb structure (PDB code 3RVY) as the search template. The data sets were processed in C2 space group and there are four molecules in one asymmetric unit. We chose the f222 space group to process the data sets for initial structural determination, but we found that the bound ions were slightly off-centre with respect to the axis of the space group and there are four molecules in one asymmetric unit. We chose the 222 space group. Crystallography and NMR System software was used for refinement of coordinates and B-factors. Final models were obtained after several cycles of refinement with REFMAC and PHENIX and manual re-building using COOT. The geometries of the final structural models of CaVAb and its derivatives were verified using PROCHECK. The divalent cations were identified by anomalous difference Fourier maps calculated using data collected at wavelengths of 1.75 Å for Ca2+, Cd2+ and Mn2+. Detailed crystallographic data and refinement statistics for all the constructs are shown in Supplementary Table 1. All structural figures were prepared with PyMol.

Electrophysiology. Wild-type NavAb expressed by infection of insect cells (High5) activates at very negative potentials (V/12 ≈ −98 mV) and shows a strong, late use-dependent phase of slow inactivation. Mutation N49K shifts the activation curve ~75 mV to more positive potentials and abolishes the use-dependent inactivation.