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MOLECULAR DETERMINANT FOR SPECIFIC Ca/Ba SELECTIVITY PROFILES OF LOW AND HIGH THRESHOLD Ca\(^{2+}\) CHANNELS

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Running title: Divalent cation selection in LVA/HVA Ca\(^{2+}\) channels

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ABSTRACT.

Voltage-gated Ca\(^{2+}\) channels (VGCC) play a key role in many physiological functions by their high selectivity for Ca\(^{2+}\) over other divalent and monovalent cations in physiological situations. Divalent/monovalent selection is shared by all VGCC and is satisfactory explained by the existence, within the pore, of a set of four conserved glutamate/aspartate residues (EEEE locus) coordinating Ca\(^{2+}\) ions. This locus however does not explain neither the choice of Ca\(^{2+}\) among other divalent cations nor the specific conductances encountered in the different VGCC. Our systematic analysis of high- and low-threshold VGCC currents in the presence of Ca\(^{2+}\) and Ba\(^{2+}\) reveals highly specific selectivity profiles. Sequence analysis, molecular modeling and mutational studies identify a set of non-conserved charged-residues responsible for these profiles. In HVA (High Voltage Activated) channels, mutations of this set modify divalent cation selectivity and channel conductance without change in divalent/monovalent selection, activation, inactivation and kinetics properties. The Ca\(v\)2.1 selectivity profile is transferred to Ca\(v\)2.3 when exchanging their residues at this location. Numerical simulations suggest modification in an external Ca\(^{2+}\) binding site in the channel pore directly involved in the choice of Ca\(^{2+}\), among other divalent physiological cations, as the main permeant cation for VGCC. In LVA (Low Voltage Activated) channels, this locus (called DCS for Divalent Cation Selectivity) also influences divalent cations selection, but our results suggest the existence of additional determinants to fully recapitulate all the differences encountered among LVA channels. These data therefore attribute to the DCS a unique role in the specific shaping of the Ca\(^{2+}\) influx between the different HVA channels.

KEYWORDS: Anomalous-mole-fraction / EEEE locus / Permeability /Molecular modeling/ HVA channels, LVA channels.
INTRODUCTION

Low (LVA= CaV3.1-3) and high-(HVA= CaV1.1-4, CaV2.1-3) threshold voltage-gated Ca\(^{2+}\) channels, by allowing selective, rapid and large Ca\(^{2+}\) influx into cells, are key actors of the Ca\(^{2+}\) homeostasis (Isom et al., 1994; Catterall, 2000) and thus play a fundamental role in cell physiology by activating a myriad of Ca\(^{2+}\)-driven processes. Although channel-specific gating, localization, regulation and protein partners, finely tuned this Ca influx and thus directly participate to the precise channel physiological or pathophysiological roles, the key feature that makes these channels so important is undoubtedly their capacity to select Ca\(^{2+}\) ions among other physiological monovalent and divalent cations. The channel preference for Ca\(^{2+}\) versus the monovalent Na\(^{+}\) has been the subject of a number of studies [(Cibulsky and Sather, 2003; Sather et al., 1994; Sather and McCleskey, 2003; Varadi et al., 1999)] mostly performed on the prototypal HVA CaV1.2 channels with only sparse data available on other channel types (Sather et al., 2003; Talavera et al., 2001; Serrano et al., 2000).

The mechanism of Ca\(^{2+}\) ion selection and permeation relies on the pore-forming Ca\(_{\alpha}\) subunit. Ca\(_{\alpha}\) subunits are composed of 4 domains, each containing 6 transmembrane segments and a pore-forming loop (P loop), arranged around a pseudo-symmetrical axis forming a central pore. Theoretical analysis and experimental finding, performed on CaV1.2 channels, have suggested that the basis of selection and high transfer rate of Ca\(^{2+}\) ions relies on direct interactions of two ions with specific residues in the pore (Hess and Tsien, 1984; Sather et al., 2003; Varadi et al., 1999). Molecular studies identified a single site, the EEEE (EEDD in LVA channels) locus, constituted by a ring of 4 glutamates (2E + 2D in LVA channels) located at equivalent position in each of the P-loop of the 4 domains (* in Fig. 2), and responsible for the multi-ionic nature of the Ca\(^{2+}\) channel pore (Yang et al., 1993; Cibulsky and Sather, 2000; Ellinor et al., 1995; Kim et al., 1993; Tang et al., 1993; Heinemann et al., 1992). The binding of one or 2 Ca\(^{2+}\) ions in the EEEE locus can explain the apparent antagonism between high permeability and high selectivity typical of these Ca\(^{2+}\) channels (Sather et al., 2003). However several studies indicate VGCC-specific selectivities for divalent cations with Ca/Ba permeability ratios varying from 0.3 to 1.2 for CaV1.2 and CaV2.3 for example (Bourinet et al., 1996), suggesting that the choice of Ca\(^{2+}\), crucial for cell physiology, cannot be explained by this conserved EEEE
locus. Other motifs known to contribute to channel permeation (Feng et al., 2001) are conserved among HVA channels and thus are not good candidates to explain channel-specific properties. Furthermore, mutation of the two Asp present at the EEDD locus of all LVA channels to Glu, as in HVA, cannot bestow HVA-like selectivity profiles (Talavera et al., 2001). Clearly, additional mechanisms and/or loci may exist to fully explain Ca$^{2+}$ selection.

We have performed the first analysis of the relative permeability to Ba$^{2+}$ and Ca$^{2+}$ ions of 3 LVA and 4 HVA VGCCs expressed in Xenopus oocytes. Specific permeation profiles are detected for each channel by characterizing the anomalous mole fraction effect (AMFE). Inspection of the amino-acid sequences of these channels in the pore region reveals a novel and channel-specific locus composed of non-conserved charged residues. In HVA channels, modifications of this site strongly influence the Ba$^{2+}$/Ca$^{2+}$ permeability ratio without modifying the monovalent/divalent selection and the sensitivity to Cd$^{2+}$ block. Our results, completed with numerical simulation and molecular modeling, suggest that this set of amino acids (thereafter called DCS=divalent cation selection), is located in the upper half of the channel and points toward the pore, where it may form a second Ca$^{2+}$ binding site. In LVA channels, mutation of the DCS also influences channel selectivity, although additional sites clearly exit. DCS is present in all VGCC and is crucial for the definition of their specific permeability profile, and thus for Ca$^{2+}$ entry into cells.
MATERIALS AND METHODS

Molecular biology. Mutations in rat brain CaV2.1 and CaV2.3 were introduced by Polymerase Chain Reaction (PCR)(GenBank acc. nb. #M64372 and #L15453 respectively) Mutations T264D, T264A, T264G, E612S, E612A, E612G, G1323F, Q1328E, Q1328A, Q1328G, S1611C, E1619N, E1619A, E1619G, E1619D and QE1618-1619HN were introduced by Polymerase Chain Reaction (PCR) mutagenesis into the rbEII sequence (CaV2.3, GenBank acc. nb. #15453). PCR fragments were then sub-cloned into a modified version of the pBluescript cloning vector, using native (NotI, XbaI, Bst98I, ApaI, HindIII, XhoI) or created (Mlu(1007), KpnI(poly linker)) restriction sites, and fully sequenced. Full length cDNA were constructed by ligating appropriate fragments. Mutations are written explicitly in the text. The same approach has been followed to construct CaV2.1(TEQE) (rbAI, #M64373). In this case, an additional mutation (histidine at position -1, referred to the DCS locus, into glutamine) was also made to preserve the pore structure of CaV2.3 (see Fig. 2). The reverse mutation (Q to H) was made on the CaV2.3 (DEQN). Finally, CaV2.3, CaV2.1, CaV1.2 (#M67515) CaVβ2a (#M80545) and CaVα2-δ (#M86621) were sub-cloned into the pMT2 expression vector for oocyte injection and expression.

Electrophysiology. Isolated Xenopus laevis oocytes were injected with a mixture of plasmids encoding the α2-δ + β2 and each of the mutant CaV2.1 or CaV2.3 Ca2+ channel subunits at a total DNA concentration of 0.2 µg/µl. LVA CaV3.1, CaV3.2, CaV3.3 subunits were injected alone. Total whole-cell currents were recorded under two-electrodes (resistance: 0.5-1 MΩ in KCl 3 M) voltage-clamp (geneclamp 500, Axon Instruments Inc, Union city, Ca) using solutions of increasing Ca2+ mole fraction (in mM: BaOH/CaOH: 10/0, 9/1, 8/2, 6/4, 4/6, 2/8, 0/10; TEAOH, 20; CsOH, 2; NMDG, 50; HEPES, 10, pH set to 7.2 using methane sulfonic acid). Voltage ramps from -80 mV to +80 mV (at 0.4 V/s) or -100 mV to +60 mV (at 0.4 to 2.6 V/s) were applied every 15 seconds on HVA or LVA VGCC expressing oocytes, respectively. The speed of the ramp was chosen after experimental testing to minimize the error in the current-voltage curve when channel inactivation was fast (CaV3.1, CaV3.2). Oocytes were injected with 40 nl of a 100 mM BAPTA solution to block contaminating Ca2+-activated Cl current. In Fig 5, the extent of AMFE was quantified by dividing the minimum of the current–mole fraction curve by the
current recorded at 10 mM Ca\textsuperscript{2+}. This allow to visualize the presence of AMFE (values <1) and the extent of the Ca/Ba current ratio on the same graph. This is not valid for channels without AMFE and with Ca\textsuperscript{2+} current larger than Ba currents, a case not present in HVA channel. The correlation between the number of charges and the extent of AMFE was tested using the Spearman correlation test at the 0.05 level.

Single-channel recordings were obtained in cell-attached patch with a bath solution composed of (in mM) KCl, 100; MgCl\textsubscript{2}, 2; HEPES, 10; EGTA, 10 pH set to 7.4 using KOH. The pipettes (10-20 M\text{\ohm}) were filled with (in mM): BaCl\textsubscript{2}, 100, HEPES, 10; pH set to 7.2 using NaOH. Currents were filtered at 2 kHz and digitized at 10 kHz. Channel conductances were calculated from linear regressions of single channel amplitudes obtained at different pipette voltages and determined using idealized event histograms (Clampfit, ver 9.0, Axon Instruments Inc.). All values are presented as mean ±SEM. Bay-K 8644 (1µM) was added to the pipette solution for the recordings obtained on the Ca\textsubscript{V}1.2 channels.

**Molecular modeling.** The helices P and S6 being located in the hydrophobic interior of the structure have highly apolar sequences and connected by a variable hydrophilic link. This motif was identified in each of four Ca\textsuperscript{2+} channels domains and aligned with the sequence of KvAT by using CLUSTALW program (Higgins et al., 1996). The sequence alignment was then manually corrected whenever necessary based on the available crystal structure of K\textsuperscript{+} channel (Jiang et al., 2003). The structure of the K\textsuperscript{+} channel provides several constraints that we used to model the 3D structure of Ca\textsuperscript{2+} channel. The C-terminal ends of four helices P come in direct contact in the tetramer centre. This arrangement requires a perpendicular exit from the \(\alpha\)-helical C-terminal cap (Efimov, 1993) induced by the left-handed \(\alpha\)-helical conformation of Thr196. In the alignment that was used for the modeling, the hydrophobic region of the Ca\textsuperscript{2+} channel corresponding to the helix P is frequently followed by glycine (methionine in domain I). Glycine following the \(\alpha\)-helix in the left-handed \(\alpha\)-helical conformation is the typical case of the perpendicular exit from the \(\alpha\)-helix (Efimov, 1993). Therefore, these glycines were aligned with Thr196 of KvAT. The next five residues represent a pore-lining region (pink bar on supp Fig. 2B). In the alignment, the residues of the EEEE and DCS loci of the Ca\textsuperscript{2+} channel are within the pore-lining region. Furthermore, an aromatic Tyr199 of the K\textsuperscript{+} pore-lining region that faces the hydrophobic interior of the
structure (marked by an arrow in supp figure2B) is aligned with the conserved aromatic tryptophans of the Ca\(^{2+}\) channel. The following loop between the pore-forming region and \(\alpha\)-helix S6 moves away from the pore and can be variable (broken line on supp Fig. 2B). Therefore, in the sequence alignment these regions are hydrophilic and of different length. The initial model containing helices P and S6 was built by using the HOMOLOGY module of InsightII program (Dayring et al., 1986) and the alignment shown on supp Fig. 2B. Then the pore-lining region of the Ca\(^{2+}\) channel was modeled manually taking into consideration the following constraints: (i) all four loops have the same backbone conformation, (ii) the side chains of the EEEE and DCS loci are directed towards the Ca\(^{2+}\) ions, as suggested by effects of the mutations and (iii) the aromatic tryptophans occupy the same hydrophobic pocket of the helices P and S6 as the aromatic Tyr199 in the K\(^+\) channel. The conformational analysis showed that to meet all these conditions the pore lining region of the Ca\(^{2+}\) channel requires a conformation that is different from one of the K\(^+\) channel. In our model (Fig 2B), this region has a conformation of the polyproline helix instead of an alternation of left-handed and right-handed alpha-helical conformations observed in the K\(^+\) channel. Two Ca\(^{2+}\) ions were introduced in the EEEE and DCS locus and side chains of the locus were manually adjusted to coordinate the cations. The final structure was energy minimized using InsightII program (Dayring et al., 1986). The energy minimisation and molecular dynamics calculations of our model suggested that the side chains of the EEEE locus coordinating Ca\(^{2+}\) are packed tighter than the ones of the DCS locus and this can explained the higher affinity of the EEEE site. The superposition of the energy minimized Ca\(^{2+}\) channel model with the structure of K\(^+\) channel shows that the subunits of Ca\(^{2+}\) channel move away from the center of the pore to allow the sides chains of EEEE locus to accommodate the pore.

Numerical simulation. The two binding-sites model for calcium channels was essentially derived from (Campbell et al., 1988; Begenisich and Cahalan, 1980; Hess et al., 1984; Kurata et al., 1999) with minor changes in the energy profiles to account for the specific permeation properties of Ca\(_{v}2.3\). The energy profile of LVA channels was obtained from that of Ca\(_{v}2.3\) by slight adjustment of the binding energy of the internal well (the EEEE/EEDD locus) to provide current traces compatible with Ca\(_{v}3.1\) currents. These two
profiles served then as references and all subsequent computations were made by varying only the binding energy of the first extracellular well (DCS locus) that was supposed to be modified by the mutations. The 3 energy peaks (P1, P2, P3) and the two wells (DCS and EEE) were regularly spaced within the membrane at similar electrical distances (Rosenberg and Chen, 1991). The membrane potential (Vm) is supposed to fall across the narrow region of the pore. The rate constants for the transitions between the different channel states were calculated by Eyring rate theory as rate=kT/h exp (-ΔG), where ΔG is the sum of the chemical energy, given by the energy differences between the peak and the well, the electric energy, given by the movement of the cation through the electrical field (zVm/d; d electrical distance) and an electrostatic repulsive force due to occupancy of two binding sites by two cations (zi; j ln(F)) see (Campbell et al., 1988; Begenisich et al., 1980; Hess et al., 1984; Kurata et al., 1999). The energy profiles for Ca2+ and Ba2+ were (in RT units) 4, -19.8, -7.5, -17.5, 5 and 4, -17.4, 1, -15.5, 5.2 for LVA and 4, -19.5, -7.5, -18.1, 1 and 4, -14.5, 1, -14.7, 2 for HVA channels. Extracellular and intracellular total divalent cation concentrations were set to 10 mM and 0.1 µM respectively. Computation of steady-state ionic currents at different Ca2+ mole fractions and decreasing DCS locus energy (-20 to -12 RT units) was made by using the matrix inversion method to solve the nine differential equations of the occupancy probabilities for the nine possible states of the model. ICa/IBa was computed as the ratio of the currents calculated in 10 mM pure Ca2+ and Ba2+ respectively. The program was written using Matlab (the Mathworks Inc.).

Online supplemental materials:
Supplementary tables I, II and III: voltage-dependent properties of CaV2.1 and CaV2.3 mutant channels.
Supplementary figure 1: comparative analysis of ramp-versus-step voltage to record AMFE
Supplementary figure 2: Compilation of selective permeation profile and amino-acid alignment of the pore region of CaV2.3 and Kv channels
Supplementary figure 3: Activation and inactivation curves of CaV2.1 and CaV2.3 mutant channels
Supplementary figure 4: Effect of non-DCS mutations on AMFE
RESULTS

One of the major theoretical outcomes of the two Ca$^{2+}$ binding-site model for Ca$^{2+}$ channel permeation is the presence of AMFE, where, in the presence of mixtures of Ba$^{2+}$ and Ca$^{2+}$ ions at a total constant concentration, the measured peak-current/mole fraction curve is non-monotonic and goes through a minimum (Hess et al., 1984). AMFE was first described for L-type VGCC (Hess et al., 1984; Almers et al., 1984). AMFE was also later reported for P/Q-type CaV2.1 channels (Mangoni et al., 1997) and expected to be very weak, from theoretical considerations, in CaV3.1 channels (Serrano et al., 2000). However, a complete comparative analysis of the Ba/Ca selectivity and AMFE of all LVA and HVA channels has never been done.

Current-voltage curves were recorded on Xenopus oocytes expressing different LVA and HVA VGCC under extracellular ionic conditions that allowed the detection of AMFE and the characterization of the Ba/Ca permeability. As shown on the current traces recorded during voltage ramps in the presence of 10/0, 9/1, 8/2, 6/4, 4/6, 2/8, and 0/10 Ba/Ca mole fractions (total cation concentration kept constant at 10 mM), L-type (CaV1.2), P/Q-type (CaV2.1), N-type (CaV2.2) but also R-type (CaV2.3) HVA Ca$^{2+}$ channels displayed a marked AMFE, with a characteristic (and statistically significant) minimum peak current value for low Ca$^{2+}$ mole fractions (current traces and peak current-Ba/Ca mole fractions curves are shown on Fig 1A and C). These channels nevertheless displayed different relative amplitudes of their Ca$^{2+}$ and Ba$^{2+}$ currents at 10 mM (ICa/IBa), ranging from 0.31±0.01 (n=23) for CaV1.2, 0.41±0.02 (n=8) for CaV2.2, 0.51±0.02 (n=17) for CaV2.1; to 1.12±0.03 (n=13) for CaV2.3; Fig 1C), suggesting specific permeation profiles despite conserved EEEE locus. Similar results were also found when current-voltage curves were obtained from voltage-steps instead of voltage-ramps demonstrating that these differences were not due to differences in Ca$^{2+}$-dependent inactivation among the different VGCC (suppl. Fig 1).

Surprisingly, when LVA T-type CaV3.1, CaV3.2 or CaV3.3 VGCC were expressed in oocytes and submitted to the same experimental protocol (Fig 1B and C), AMFE was only recorded for CaV3.3, while peak current decreased, or increased, monotonically with increasing Ca$^{2+}$ mole fractions for CaV3.2 and CaV3.1 channels, respectively. These new results were completely unexpected since these 3 channels display the same sequence at
their EEDD locus. Moreover, all 3 channels display a specific Ba/Ca permeability with either larger (ICa/IBa=1.91±0.06, n=10 for CaV3.1), smaller (ICa/IBa= 0.67±0.02, n=8 for CaV3.2) or similar (1.23±0.02, n=5 for CaV3.3) Ca\(^{2+}\) currents relative to Ba\(^{2+}\) currents.

When compiled together these data unambiguously underlined channel-specific permeation profiles in the two channel families (see supp. Fig 2A). Such a specificity cannot be explained by the only high-affinity Ca\(^{2+}\) binding-site identified, which is perfectly conserved in these 2 families (the EEEE and EEDD loci in HVA and LVA channels respectively, see * box in Fig 2A) (Sather et al., 2003; Cibulsky et al., 2000). Inspection of the aligned amino-acid sequences (Fig 2A) of the pore-forming P-loops of HVA and LVA channels reveals the existence of a single set of non-conserved negatively charged residues located at homologous position in each of the four domains of the Ca\(_{\alpha1}\) subunits, 4 residues upstream of the well characterized EEEE/EEDD locus (# box in Fig 2A). This DCS locus comprises amino acids DSED, DEQN, DAME and TEQE in the channel domains I-IV respectively, in CaV1.2, CaV2.1, CaV2.2 and CaV2.3 and amino acids DKDG, DVNG and EVNG in CaV3.1, CaV3.2 and CaV3.3 channels.

The lack of structural data on the pore of the VGCC imposed the use of molecular modeling to visualize the possible arrangement of the 4 CaV2.3 P-loops at the EEEE and DCS loci. This exercise has been done using voltage-gated K\(^{+}\) channel as template (Doyle et al., 1998; Jiang et al., 2003) with major constraints that take into account the fundamental differences that exist between the K\(^{+}\) and Ca\(^{2+}\) channel pores; see Fig 2B, and supp. Fig 2B (Lipkind and Fozzard, 2001; Hille, 2001). The DCS locus is located on the extracellular side of the EEEE locus but likely within the membrane electric field as suggested (Hess et al., 1984) and with inter-loci distances of around 8 Å. The carboxyl oxygen’s of the DCS locus can point towards the channel lumen, allowing their participation in the coordination of incoming divalent cations. Thus, from theoretical and experimental consideration on AMFE, the DCS locus constitutes, in several respects, a good candidate for a specific role in divalent cation selection.

Although all residues at this locus may not play an equivalent role, as is the case at the EEEE locus (Yang et al., 1993; Parent and Gopalakrishnan, 1995; Cloues et al., 2000; Tang et al., 1993; Mikala et al., 1993; Kim et al., 1993), our first hypothesis was that the
total number of negatively charged residues at this location was critical for the binding of Ca\(^{2+}\) ions.

To test this hypothesis, 19 mutations were engineered in the DCS locus of Ca\(_V\)2.3, where the number of negatively charged residues was either, increased (DSEE, DSED and DEEE), or decreased (TSQN, GGGG or AAAA). The resulting 6 mutants, labeled Ca\(_V\)2.3(DSEE), Ca\(_V\)2.3(DSED), Ca\(_V\)2.3(DEEE), Ca\(_V\)2.3(TSQN), Ca\(_V\)2.3(GGGG) and Ca\(_V\)2.3(AAAA) respectively (Fig 3), were tested for their effect on channel selectivity. Currents recorded from Xenopus oocytes injected with the Ca\(_V\)2.3(DSED) mutant cDNA showed a marked minimum for Ba/Ca mixtures of low Ca\(^{2+}\) mole fractions, and pure Ca\(^{2+}\) currents were clearly smaller than pure Ba\(^{2+}\) currents (ICa/IBa=0.60±0.05, n=16, circle, Fig. 3A and B). This type of behavior was typical of Ca\(_V\)1.2 channels, which also possessed 3 negative charges at the DCS locus. Similar results were also found with the Ca\(_V\)2.3(TSQN) mutant (hexagon in Fig 3A and B), with no negative charges at the DCS, had also a smaller ICa/IBa ratio (0.44±0.02, n=11) but in this case the AMFE completely disappeared and the total current became a monotonic function of the Ca\(^{2+}\) mole fraction, without any marked minimum. Similar results were also found when the 4 residues of the DCS locus were mutated to alanine or glycine (Ca\(_V\)2.3(AAAA) or Ca\(_V\)2.3(GGGG) in Fig 3) revealing important modifications in the energy profile of the channel pore (Hess et al., 1984) clearly dependent upon the net charge carried by the residues at the DCS. Interestingly, the Ca\(_V\)2.3(DEEE) mutant, with 4 charged residues at the DCS locus had an intermediate behavior, with a clear minimum in the current/mole fraction curve and an ICa/IBa ratio of 1.02±0.05 (n=15) between that of Ca\(_V\)1.2 and Ca\(_V\)2.3 (Fig. 3B, see discussion).

As a putative structural determinant of VGCC selectivity filter, mutations in the DCS locus are expected to modify permeation and single-channel conductance. In cell-attached patches performed in 100 mM Ba\(^{2+}\), the single channel conductance of Ca\(_V\)2.3(DSED) (21±1pS, n=9, Fig 3C) and Ca\(_V\)2.3(DSEE) (22±1pS, n=7, traces not shown) channels were clearly larger than the wild type Ca\(_V\)2.3 (13±1pS, n=8) and closely matched that of Ca\(_V\)1.2 channels (22±2pS, n=8, Fig 3C) without any obvious changes in the channel open time (not shown but see WT and mutated Ca\(_V\)2.3 current traces in Fig 3C). The Ca\(_V\)2.3(AAAA) single channel amplitude was on the opposite much smaller with only
Our macroscopic recordings confirmed that, for the 6 mutated channels studied here, as for the others described below, the changes in selectivity occurred with no other modification in the biophysical properties of the channel, including current-voltage and steady-state inactivation curves (supp. Fig. 3A, B and supp. Tables II and III). The kinetics of inactivation, estimated by the fractional current recorded at the end of a 2.5 s long pulse (to +10 mV) in Ba\(^{2+}\) and in Ca\(^{2+}\), were also not modified by these mutations (supp. Fig 3C). Moreover, Cd\(^{2+}\) block of Ba\(^{2+}\) currents and current reversal potentials in 10 mM Ba\(^{2+}\) and 10 mM Ca\(^{2+}\) were only marginally affected (supp. Table I), demonstrating that mutations at the DCS have localized effects that did not spread through the protein via global perturbation of the channel gating, the pore structure, or the EEEE locus.

To challenge the idea that the DCS was involved in the specificity of the divalent cation selectivity profiles of HVA Ca\(^{2+}\) channels, we decided to switch the DCS of 2 related channels (Ca\(_V\)2.1 and Ca\(_V\)2.3) and analyze the selectivity profiles of the resulting channels.

Currents recorded from oocytes expressing Ca\(_V\)2.3(DEQN) mutated channels, (with Ca\(_V\)2.1 DCS implemented in Ca\(_V\)2.3) displayed a strong AMFE, with a clear minimum recorded for small Ca\(^{2+}\) mole fractions and ICa/IBa ratio close to that of Ca\(_V\)2.1 channels (0.66±0.02, n=11). Conversely, currents recorded from oocytes expressing Ca\(_V\)2.1(TEQE) Ca\(^{2+}\) channels (with Ca\(_V\)2.3 DCS introduced in Ca\(_V\)2.1) displayed AMFE and IC/IBa ratio similar to those of Ca\(_V\)2.3 channels (ICa/IBa=0.92±0.02, n=30, Fig 4A and B). Again, these modifications occurred without changes in the divalent over monovalent selectivity, (Ca\(^{2+}\) or Ba\(^{2+}\) current reversal potentials were not modified, Fig 4C), and in the channel affinity for Cd\(^{2+}\), the small differences in the EC50 for Cd\(^{2+}\) block between Ca\(_V\)2.3 and Ca\(_V\)2.1 being preserved (Fig. 4C). Thus, mutations at the DCS locus only affected divalent selectivity without affecting the EEEE locus and EEEE-related properties (divalent/monovalent selectivity and Cd\(^{2+}\) binding site). These data suggested that DCS forms a distinct locus involved in the multi ionic nature of the channel selectivity filter (Kuo and Hess, 1993a; Kuo and Hess, 1993b).

To summarize the effects of these different HVA Ca\(^{2+}\) channel mutations on Ba\(^{2+}/Ca^{2+}\) selection, we have plotted the extent of AMFE (in arbitrary units, see methods)
as a function of the number of negative charges at the DCS. AMFE values close to 1 indicate no mole fraction, while smaller values indicate the presence of stronger AMFE with larger Ca/Ba current ratios. As shown in Fig 5, a clear and statistically significant relation between charges and AMFE exits (using the Spearman correlation test at a 0.05 level that makes no assumption on the nature of the relation). Although care should be taken in interpreting such graph because AMFE and ICa/IBa ratios clearly not vary linearly with ion binding energy (see Fig 7), this nevertheless confirms the implication of the DCS locus on HVA channels selectivity.

It is worth noting that the conservation of the EEEE locus among HVA-VGCC does not exclude a crucial role in divalent selection but rather conservation of this role in the VGCC family. To test this idea, we mutated a non-conserved glycine residue on Cav2.3 that has been shown to slightly decrease Ca$^{2+}$ binding at the EEEE locus and perturb channel permeation (Williamson and Sather, 1999) and analyzed the effects of this mutation on AMFE and Ca/Ba selectivity. The mutant Cav2.3(G1323F) channels displayed a decreased in AMFE and in relative Ca$^{2+}$ currents (ICa/IBa) in close proportion with the reported decrease in the binding energy for divalent cation (Williamson et al., 1999) at the EEEE locus (ICa/IBa=1.12±0.03, n=13 and 0.70±0.05, n=10 for Cav2.3 and Cav2.3(G1323F) respectively, supp. Fig 4A and B) confirming the role of the EEEE locus in profiling channel permeation. On the other hand, mutation of a more distant non-conserved residue outside these two loci (S1611C) had no effect neither on Cav2.3 AMFE nor on the relative Ca$^{2+}$ currents (ICa/IBa=1.12±0.03, n=13 and 1.06±0.05, n=6 for Cav2.3 and Cav2.3(S1611C) respectively, supp Fig 4A and B). This mutation had also no effect when made on the Cav2.3(DSED) mutant channels with ICa/IBa=0.60±0.05, n=16 and ICa/IBa=0.60±0.08, n=9 for Cav2.3(DSED and Cav2.3(DSED)(S1611C)).

Having in mind the large differences in the current-mole fraction curves between the different LVA channels, we have mutated the DCS locus of Cav3.2 (DCS: DVNG, see Fig 6A) to match that of either Cav3.1 or Cav3.3 (DCS locus: DKDG or EVNG, respectively) giving rise to the two mutated Cav3.2 channels: Cav3.2(DKD) and Cav3.2(EVNG). Current-mole fraction curves obtained with these 3 channels are displayed in Fig 6B, and clearly showed that the mutation of the Cav3.2 DCS to EVNG had no marked effect on AMFE and on the relative Ca$^{2+}$ currents, while mutation to DKDG clearly modified these
two parameters with the appearance of a small AMFE, absent in the parent Ca\textsubscript{V}3.2 channels (curves of Ca\textsubscript{V}3.1 and Ca\textsubscript{V}3.3 channels are in dashed lines for comparison). As was the case for HVA channels, these changes appeared without any modification in the current-voltage and steady-state inactivation curves of the channels, the potential of half-activation, the potential for half-inactivation, the slope the two curves and the reversal potential remaining unchanged (Fig 6C). The kinetics of the currents was also not modified (not shown). These data suggest that the modifications recorded with the Ca\textsubscript{V}3.2(DKDG) mutant are not due to perturbations of the LVA EEDD locus, known to change reversal potential and current kinetics (Talavera et al., 2003; Talavera et al., 2001).

Altogether, these results suggest that in HVA channels, the differences in divalent cation permeation recorded between voltage-gated Ca\textsuperscript{2+} channels were mainly due to variations in the structure of the DCS and EEEE loci, while in LVA channels, although these two sites are also implicated, they may not be the only determinants.
DISCUSSION

This first detailed analysis of the divalent cation selectivity of LVA and HVA VGCC identified a ring of non-conserved negative charges in the upper part of the pore directly involved in the selection of Ca\(^{2+}\) by Ca\(^{2+}\) channels. Modifications of the pattern of charged residues at this site affect both AMFE and relative Ca\(^{2+}\) current in HVA and LVA VGCC.

Previous work on Ca\(_V\)1.2 (Cibulsky et al., 2000; Ellinor et al., 1995; Sather et al., 2003) strongly suggested that high-affinity Ca\(^{2+}\) binding in the VGCC pore involved a single site: the EEEE locus. Although a role for the EEEE locus in divalent selection makes no doubt, its implication in the specificity of the selection observed here, via a global perturbation of the channel structure by DCS mutations, seems very unlikely for several reasons. First, the conservation of the pattern of charged residues at the EEEE/EEDD locus on HVA/LVA channels, excludes this locus as a major determinant for channel-specific properties. Second, properties known to be strongly modified by mutation at the EEEE locus (Ba\(^{2+}\) or Ca\(^{2+}\) current reversal potentials, Cd\(^{2+}\) inhibition) are not changed by similar mutation at the DCS. Third, DCS mutations have no side effects on the voltage-dependent properties or on the current kinetics. On the opposite, we can speculate that quadruple mutations at internal EEEE locus (Cibulsky et al., 2000; Ellinor et al., 1995) may destabilize the less-conserved DCS locus and reduce Ca\(^{2+}\) binding at this site. Of course, our results on the Ca\(_V\)2.3(G1323F) mutant demonstrate that the EEEE locus participates at the global shaping of these permeation profiles, but not at their specificity encountered in the different VGCC.

We propose that the number of charged residues present at the VGCC DCS locus is critical for the binding of Ca\(^{2+}\) ions. Three negative charges are present in the Ca\(_V\)1.2 and Ca\(_V\)2.3(DSED) channels which display the highest Ba\(^{2+}\) conductance and the lowest ICa/IBa ratio. With only 2 negative charges, Ca\(_V\)2.1(DCS=DEQN), Ca\(_V\)2.2 (DCS=DAME) and Ca\(_V\)2.3 (DCS=TEQE) have lower Ba\(^{2+}\) conductances, but higher ICa/IBa ratios. The differences in the permeation profile that remains between these 3 channels may rely (1) on the non-charged residues present at this site (i.e. glutamines are known to be able to participate in Ca\(^{2+}\) binding (Kim et al., 1993)), (2) on non-equivalent positioning of these two charges within the 4 channel domains and/or (3) on other neighboring amino-acids that
could finely shape the structure of the DCS. Although the characterization of the precise role of these 3 factors will require further experiments, it is worth noting that the effects of the mutation of glutamate at the EEEE or EEDD loci in HVA and LVA channels are known to be strongly dependent of their location (Sather et al., 2003; Mikala et al., 1993; Talavera et al., 2001).

Different theoretical models have been proposed (Hess et al., 1984; Sather et al., 2003; Miller, 1999; Roux, 1999) to account for the specific permeation properties of VGCC including (1) the selection of Ca over Na despite identical size and unfavorable concentration, (2) high Ca fluxes through open channel and (3) high affinity block of monovalent currents by Ca$^{2+}$. In our case, we thought modeling the Ca$^{2+}$ channel as a multi-ion single file pore with two Ca$^{2+}$ binding sites and 3 energy barriers (Hess et al., 1984) was particularly appropriate to understand the role of rings of negative charges in the channel lumen (Fig 7A). AMFE was computed using rate transitions calculated from Eyring rate theory with energy profiles for Ca$^{2+}$ and Ba$^{2+}$ derived from previous works (Hess et al., 1984; Campbell et al., 1988). Current/mole fraction curves were obtained by sequentially decreasing the binding energy ($\Delta G^\circ$) of the external locus. For large $\Delta G^\circ$ at the DCS (as expected for Ca$_V$1.2 channels), the ICa/IBa ratio was around 0.3 and total current showed AMFE with a strong reduction for small Ca$^{2+}$ mole fractions (Fig. 7B). When $\Delta G^\circ$ was progressively decreased, keeping all other parameters constant, ICa/IBa reached a maximum at around 1.5 (close to wild-type Ca$_V$2.3 channels) and then progressively returned to lower values. For these values however, the current decreased monotonically and did not display AMFE (Fig. 7B and (Hess et al., 1984)) similar to recordings obtained from the Ca$_V$2.3(TSQN) Ca$_V$2.3 (AAAA) or the Ca$_V$2.3 (GGGG) channels without negative charges at the DCS. Whether one or multiple Ca$^{2+}$ ions remain bound to the EEEE locus in these mutants devoid of AMFE is an open question that requires further experiments.

Our simulation also suggest that Ca$_V$2.3 (DEEE) mutant, with strong AMFE but ICa/IBa ratio close to the parent Ca$_V$2.3 channel, despite 2 additional negative charges at the DCS locus may result from DCS with a smaller $\Delta G^\circ$ then Ca$_V$1.2 (Fig. 7B). This can be explained by an increase in the electrostatic repulsion generated by the 2 additional carboxylates present of the DCS locus (Wu et al., 2000) leading to looser cations coordination. Mimicking the decrease in the $\Delta G^\circ$ of the EEEE locus for the Ca$_V$2.3(G1323F)
mutant (by 1 RT units see (Williamson et al., 1999)) also resulted in computed current-
mole fraction curves (supp Fig. 4B) close to those obtained experimentally in terms of relative Ca\(^{2+}\) currents and amplitude of AMFE. In an interesting alternative, the DCS locus could also constitute one of the low affinity sites proposed by Dang and McCleskey (Dang and McCleskey, 1998) to be necessary to overcome the large energy barriers flanking a single high affinity binding site. However, further experimental testing is required to more definitively validate one of these models.

In the case of LVA VGCC, simulation with decreasing \(\Delta G^o\) at the DCS (Fig 7C) and comparison with experimental results (Fig 1) suggested the following sequence of \(\Delta G^o\) at DCS Ca\(_{V3.1}\)>Ca\(_{V3.3}\)>Ca\(_{V3.2}\). Calculation of single channel currents using these differences in \(\Delta G^o\) gives a higher single channel conductance for Ca\(_{V3.3}\) than for Ca\(_{V3.1}\) or Ca\(_{V3.2}\) (not shown), a situation that is recorded experimentally (Lee et al., 1999; Perez Reyes et al., 1998; Cribbs et al., 1998). However, although the DCS locus is undoubtedly involved in shaping the specific selectivity of LVA channels (see Ca\(_{V3.2}(DKDG)\) mutant), we were not able to completely restore the properties of the different Ca\(_{V3}\) channels by mutation of the DCS. The existence of non-EEEE/EEDD structural determinants involved in the differences in the selectivity recorded between HVA and LVA and among LVA channels have been postulated by Talavera et al. (Talavera et al., 2001). Our results demonstrate that DCS is one of them and clearly suggest the existence of other elements, at least for LVA channels. The strong conservation of the EEDD locus and the P-loop sequences in the different members of the LVA channels family locate these putative determinants implicated in LVA permeation at a more external position.

The presence of two sets of negative charges in the channel pore to regulate channel selectivity is a general scheme that has been conserved among different Ca\(^{2+}\) channels. An homologous external locus is also found in K\(_V\) (MacKinnon and Yellen, 1990; Crouzy et al., 2001) and Na\(_V\) channels (Hille, 2001) with important roles in permeation and pharmacology. From a physiological point of view, DCS clearly and directly participates to the generation of large Ca\(^{2+}\) influx (see Fig. 5). Its non-conservation among HVA channels plays a role in the definition of the channel-specific conductance, and thus could participate
to the adaptation of each channel to its particular function: large and global Ca\(^{2+}\) influx for
excitation-contraction coupling driven by Ca\(_{\text{v}}\)1.2, or smaller and more local Ca\(^{2+}\) changes
required for Ca\(_{\text{v}}\)2.1-triggered synaptic transmission, for example. Our preliminary data, on
Ca\(_{\text{v}}\)2.1/Ca\(_{\text{v}}\)2.3 and Ca\(_{\text{v}}\)3.1/Ca\(_{\text{v}}\)3.2 channels also suggest an implication in the differential
block of these channels by external magnesium (not shown).

In conclusion, these results demonstrate the unexpected existence of an external
locus that may form part of a structural context necessary for the pore of HVA and LVA
Ca\(^{2+}\) channels to display high Ca\(^{2+}\) influx and specific divalent cation selectivity. Whether
this site is also important for other pore-dependent properties such as toxin or drug binding,
is now under investigation.
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Footnotes:
VGCC: Voltage-gated Ca\(^{2+}\) channels; LVA: low voltage activated; HVA: High voltage activated; DCS: divalent cation selectivity, TEAOH: tetra-ethyl-ammonium hydroxide; AMFE: anomalous mole fraction effect.

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**FIGURE LEGENDS**

Figure 1. Low and High-voltage activated Ca$^{2+}$ channels have specific Ca$^{2+}$/Ba$^{2+}$ selectivities.

A,B. Representative current traces recorded in oocytes expressing HVA CaV1.2, CaV2.1, CaV2.2 or CaV2.3 and $\alpha_2-\delta + \beta_2$ Ca$^{2+}$ channel subunits (A) or LVA CaV3.1, CaV3.2 or CaV3.3 Ca$^{2+}$ channels (B) using solutions of different Ca$^{2+}$ mole fractions (Ba$^{2+}$/Ca$^{2+}$: 10/0, 9/1, 8/2, 6/4, 4/6, 2/8 and 0/10 mM). Whole-cell currents were recorded during voltage ramps from -80 to +80 mV (A) or from -100 to +60 mV (B) at a speed of 0.4 to 2.6V/s applied every 15 seconds. (Scale bars: 100 nA).

C. Normalized current/mole fraction curves obtained from recordings similar to those in A or B by plotting the peak-current of the IV curve against the Ca/Ba mole fraction. Note the different ICa/IBa ratios and the presence/absence of AMFE in each case. Left, averaged curves obtained from oocytes expressing CaV1.2 (▲), CaV2.1 (○), CaV2.2 (▽) or CaV2.3 (□) HVA VGCC. Right, curves obtained from oocytes expressing CaV3.1 (□), CaV3.2 (▲) or CaV3.3 (○) LVA VGCC.

Figure 2. Molecular modeling of the DCS and EEE loci in the channel pore

A. Sequence alignment of the P-loop of CaV1.2, CaV2.1, CaV2.3 HVA channels and CaV3.1, CaV3.2 and CaV3.3 LVA channels. * and # boxes: position of EEEE and DCS loci in each domain of the CaV$\alpha$ subunit. Note the non-conservation of the DCS charged amino-acids. **Arrows underline the non-DCS amino-acids mutating in supplementary Fig 4: S1611 and G1323 in CaV2.3.**

B. A molecular model of the C-terminal end of the 4 P-loops of CaV2.3 channel. Left: radial projection of the channel with one of the domains omitted for the clearness of the picture. Right: Schematic representation of the EEEE and DCS loci of CaV2.3 channel. Ribbons represent the backbone of the peptidic chains. Ca$^{2+}$-ions are shown as magenta balls. Negatively charged side chains of the channel are shown by ball-and-stick representation (see Materials and Methods for details).
Figure 3. Mutations at the DCS locus suppress anomalous mole fraction of HVA.
The 4 amino acids of the Ca\textsubscript{v}2.3 DCS locus were mutated by adding or removing negatively charged residues.

A. Typical current traces recorded during voltage-ramps under different ionic conditions (see Fig. 1) applied to oocytes expressing Ca\textsubscript{v}2.3 channels with their DCS loci mutated in DSED (1 negative charge added, Ca\textsubscript{v}2.3(DSED)), DEEE (2 negative charges added, Ca\textsubscript{v}2.3(DEEE)), TSQN, AAAA or GGGG (2 negative charges removed, Ca\textsubscript{v}2.3(TSQN), Ca\textsubscript{v}2.3(AAAA) or Ca\textsubscript{v}2.3(GGGG)).

B. Current-mole fraction curves obtained for the above mutations. Right: effect of adding negative charges (Ca\textsubscript{v}2.3(DSED): ○, Ca\textsubscript{v}2.3(DEEE), ▽). Left: effect of removing negative charges (Ca\textsubscript{v}2.3(TSQN): □, Ca\textsubscript{v}2.3 (AAAA): hexagon, Ca\textsubscript{v}2.3 (GGGG) ▾). Note that the removal of the negative charges suppressed AMFE in all 3 cases. The dotted line represents the curve obtained with the WT Ca\textsubscript{v}2.3 in Fig. 1.

C. Single-channel conductances of Ca\textsubscript{v}2.3, Ca\textsubscript{v}2.3(DSED), Ca\textsubscript{v}2.3(AAAA) and Ca\textsubscript{v}1.2 VGCC. Left, current traces in 100 mM BaCl\textsubscript{2} (pipette potential = +10 mV, except Ca\textsubscript{v}2.3(AAAA) -20 mV). Right, current-voltage curves with superimposed linear regressions (13±1pS, 21±1pS, 4±1pS and 22 ±1pS for Ca\textsubscript{v}2.3, Ca\textsubscript{v}2.3(DSED), Ca\textsubscript{v}2.3(AAAA) and Ca\textsubscript{v}1.2 respectively). Scale bar: 0.5 pA, 25 ms.

Figure 4. DCS locus controls the channel-specific cation selectivity profile.

A. A Ca\textsubscript{v}2.3 channel with an engineered Ca\textsubscript{v}2.1 DCS locus (Ca\textsubscript{v}2.3(DEQN), square) displays AMFE and has an IC\textsubscript{a}/IBa ratio similar to Ca\textsubscript{v}2.1. Conversely, a Ca\textsubscript{v}2.1 channel with a Ca\textsubscript{v}2.3 DCS locus (Ca\textsubscript{v}2.1(TEQE), triangle) behaves like Ca\textsubscript{v}2.3. A. currents traces and B. current-mole fraction curves. Dashed lines: current/mole fraction curves of parent channels, Ca\textsubscript{v}2.3 and Ca\textsubscript{v}2.1, determined in Fig. 1.

C. The current reversal potentials, recorded in 10 mM Ca\textsuperscript{2+} or 10 mM Ba\textsuperscript{2+} (left), and the Cd\textsuperscript{2+} block of Ba\textsuperscript{2+} current (right) for Ca\textsubscript{v}2.3 (□) and Ca\textsubscript{v}2.1(▽) channels are not changed by the mutations (Ca\textsubscript{v}2.3(DEQN), ○ and Ca\textsubscript{v}2.1(TEQE), △); suggesting the EEEE locus is not modified.
Figure 5. **AMFE amplitude is correlated with the number of negative charges at the DCS.**

Scatter-plot showing the amplitude of AMFE (calculated as the minimum of the current – mole fraction curve divided by the current in 10 mM Ca\(^{2+}\)) as a function of the number of negative charges for all the HVA mutations presented in this article.

Figure 6. **Mutations at the DCS locus modify selectivity profiles of LVA without changes in gating.**

A. Schematic representation of the amino acids present at the Cav3.1, Cav3.2 and Cav3.3 DCS loci.

B. Current-mole fraction curves obtained for the WT (△) and two mutants Cav3.2 LVA channels mimicking the DCS loci of Cav3.1 (Cav3.2(DKDG), □) and Cav3.3 (Cav3.2(EVNG), ○). Ba/Ca current ratio was significantly changed for the Cav3.2(DKDG) mutant which also displayed a small AMFE as opposed to WT Cav3.2.

C. Voltage-dependent parameters of the current-voltage curves and inactivation curves of the Cav3.2, Cav3.2(EVNG) and Cav3.2(DKDG) LVA channels. These two mutations were without noticeable effects on these parameters.

Figure 7. **Numerical computation predicts mutant behavior.**

A. Schematic representation of the energy profile used for computer modeling of the 3 barriers-2 sites channel model. Barriers (P1, P2, P3) and wells (DCS and EEEE) are spaced equally (see Materials and Methods). Membrane-voltage drops across the narrow portion of the channel.

B. **Left.** Simulated ICa/IBa ratios calculated for decreasing free energies at the external DCS locus of the Cav2.3 channel. The bell-shaped relation obtained for the DCS locus reached a maximum when DCS ∆G\(^0\) equals the energy of the internal site. **Right.** Simulated currents-mole fraction curves with 3 out of the 4 decreasing DCS ∆G\(^0\) depicted on the left. These curves were labeled Cav1.2, Cav2.3(DEEE) and Cav2.3(GGGG), after comparison with original traces shown in Fig 1 and 3, and suspected variations in free energy at DCS locus due to mutation.
C. Simulated ICa/IBa ratios calculated for decreasing free energies at the external DCS locus of the Ca\textsubscript{v}3.1 channel. Energy profile of Ca\textsubscript{v}3.1 was first adapted from Ca\textsubscript{v}2.3 by adjusting the $\Delta G^\circ$ at the EEEE locus considering that this locus in LVA channels (EEDD) has lower affinity for Ca\textsuperscript{2+}. Simulated current-mole fraction curves were then obtained by decreasing $\Delta G^\circ$ at the DCS locus. Traces were labeled after comparison with original traces shown in Fig. 1 and suspected variations in free energy at DCS for the purpose of discussion.