The EEEE Locus Is the Sole High-affinity Ca\(^{2+}\) Binding Structure in the Pore of a Voltage-gated Ca\(^{2+}\) Channel Block by Ca\(^{2+}\) Entering from the Intracellular Pore Entrance

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**Abstract**

Selective permeability in voltage-gated Ca\(^{2+}\) channels is dependent upon a quartet of pore-localized glutamate residues (EEEELocus). The EEEE locus is widely believed to comprise the sole high-affinity Ca\(^{2+}\) binding site in the pore, which represents an overturning of earlier models that had postulated two high-affinity Ca\(^{2+}\) binding sites. The current view is based on site-directed mutagenesis work in which Ca\(^{2+}\) binding affinity was attenuated by single and double substitutions in the EEEE locus, and eliminated by quadruple alanine (AAAA), glutamine (QQQQ), or aspartate (DDDD) substitutions. However, interpretation of the mutagenesis work can be criticized on the grounds that EEEE locus mutations may have additionally disrupted the integrity of a second, non-EEEELocus high-affinity site, and that such a second site may have remained undetected because the mutated pore was probed only from the extracellular pore entrance. Here, we describe the results of experiments designed to test the strength of these criticisms of the single high-affinity locus model of selective permeability in Ca\(^{2+}\) channels. First, substituted-cysteine accessibility experiments indicate that pore structure in the vicinity of the EEEE locus is not extensively disrupted as a consequence of the quadruple AAAA mutations, suggesting in turn that the quadruple mutations do not distort pore structure to such an extent that a second high affinity site would likely be destroyed. Second, the postulated second high-affinity site was not detected by probing from the intracellularly oriented pore entrance of AAAA and QQQQ mutants. Using inside-out patches, we found that, whereas micromolar Ca\(^{2+}\) produced substantial block of outward Li\(^{+}\) current in wild-type channels, internal Ca\(^{2+}\) concentrations up to 1 mM did not produce detectable block of outward Li\(^{+}\) current in the AAAA or QQQQ mutants. These results indicate that the EEEE locus is indeed the sole high-affinity Ca\(^{2+}\) binding locus in the pore of voltage-gated Ca\(^{2+}\) channels.

**Key words:** ion selectivity • selectivity filter • L-type Ca\(^{2+}\) channel • patch clamp • cysteine mutagenesis

**Introduction**

For voltage-gated Ca\(^{2+}\) channels, selective permeability is believed to involve interaction between two or more permeant ions. However, the number of pore-localized ion binding sites that may mediate these interactions has been contentious, with assertions of two or more binding sites (Almers and McCleskey, 1984; Hess and Tsien, 1984; Hess et al., 1986; Lansman et al., 1986; Friel and Tsien, 1989; Yue and Marban, 1990; Rosenberg and Chen, 1991; Kuo and Hess, 1993a,b,c), one binding site (Kostyuk et al., 1983; Kostyuk and Mironov, 1986; Lux et al., 1990; Armstrong and Neyton, 1991), or no discrete binding sites (Nonner and Eisenberg, 1998) made by various researchers. Two-site models have been the most widely considered (McCleskey, 1999; Miller, 1999), and they are similar in nature to the multi-ion, multi-site models that have been used to describe block and flux in voltage-gated K\(^{+}\) channels (Hodgkin and Keynes, 1955; Hille and Schwarz, 1978; Neyton and Miller, 1988a,b). The general validity of these kinds of multi-ion, multi-site models has been strongly supported by the recently obtained crystal structure of a bacterial K\(^{+}\) channel (Doyle et al., 1998). This structure reveals a narrow, single-file selectivity filter containing a pair of discrete sites of dehydrated metal ion localization (binding) separated by a short distance of ~7.5 Å. This small separation very likely allows significant ion-ion interaction to occur within the K\(^{+}\) channel’s pore, as has also been postulated for Ca\(^{2+}\) channels. In the case of Ca\(^{2+}\) channels, tight binding of one Ca\(^{2+}\) ion within the single-file region of the pore obstructs permeation by foreign ions (selectivity), whereas Ca\(^{2+}\)-Ca\(^{2+}\) interaction inside the pore overcomes tight Ca\(^{2+}\) binding to generate high Ca\(^{2+}\) throughput (unitary Ca\(^{2+}\) current). These two processes can be observed in an experimental setting, where the probability of pore occupancy by Ca\(^{2+}\) can be manipulated: with approximately micromolar Ca\(^{2+}\) in the bath, the probability of single occupancy by Ca\(^{2+}\) is high, so that Ca\(^{2+}\) effectively blocks monovalent metal cation (e.g., Na\(^{+}\)) flux; with approximately millimolar Ca\(^{2+}\) in the bath, the probability of double occupancy by Ca\(^{2+}\) is high, so that Ca\(^{2+}\)-Ca\(^{2+}\) interactions within the pore are frequent.
and high unitary Ca\(^{2+}\) flux is achieved (Almers and McCleskey, 1984; Hess and Tsien, 1984; Fukushima and Hagiwara, 1985; Matsuda, 1986). In a physiological setting, Na\(^{+}\) competes with Ca\(^{2+}\) for entry into the pore of a Ca\(^{2+}\) channel so that the pore fluctuates between single and double occupancy by Ca\(^{2+}\), resulting in a Na\(^{+}\)-interrupted Ca\(^{2+}\) flux (Polo-Parada and Korn, 1997). This basic description of the selective permeability of voltage-gated Ca\(^{2+}\) channels has remained largely intact up to the present, but its structural underpinnings have been revised in recent years.

Site-directed mutagenesis studies have identified a set of four conserved glutamate residues that contribute to Ca\(^{2+}\) binding, with the glutamates referred to in ensemble as the EEEE locus (Heinemann et al., 1992; Kim et al., 1993; Mikala et al., 1993; Tang et al., 1993; Yang et al., 1993; Yatani et al., 1994; Parent and Gopalakrishnan, 1995). The roles of the EEEE locus glutamates in permeant ion binding have been investigated using a systematic set of EEEE locus mutants (Ellinor et al., 1995). In these experiments, block by a high-affinity cation of current carried by a low-affinity cation was used to estimate the effects of the mutations upon binding of the high-affinity cation. Because single amino-acid substitutions for any one of the glutamates affected block, and because none of the double substitution mutants retained high-affinity binding, it was concluded that the EEEE locus forms a single high-affinity binding site for Ca\(^{2+}\). Replacement of the entire quartet of EEEE locus glutamates with alanine or glutamine residues (AAAA or QQQQ mutants) eliminated all high-affinity binding, leading to the conclusion that the EEEE locus forms the only high-affinity binding site in the pore of Ca\(^{2+}\) channels. Indeed, theoretical studies have indicated that models incorporating a single high-affinity Ca\(^{2+}\) binding site are capable of accounting for nearly all of the selective permeability properties of Ca\(^{2+}\) channels, although low-affinity flanking sites may be required as well (Armstrong and Neyton, 1991; Dang and McCleskey, 1998).

Yet the existence of a second high-affinity, pore-localized Ca\(^{2+}\) binding site has remained a lurking possibility, according to either of the following two lines of reasoning. One criticism holds that the single and double mutations introduced into the EEEE locus not only reduced ion binding affinity in that locus, but that, in addition, the mutations disrupted function of the second high-affinity binding site. Such an effect could be viewed as arising from a more extensive disruption of pore structure than is hoped for with site-directed mutagenesis, or more elaborately, as a consequence of disruption by the mutations of a hypothesized allosteric interaction between the two high-affinity sites. A second criticism focuses on the fact that the conclusions of Ellinor et al. (1995) were based upon study of block by external divalent cations, but divalent cations were also found in that work to be essentially impermeant in the AAAA or QQQQ mutants. Thus, neither Ca\(^{2+}\) nor Ba\(^{2+}\) currents are detectable in the AAAA or QQQQ mutant channels. In principle, therefore, the pore might possess a second high-affinity binding site internal to the EEEE locus, but in the AAAA and QQQQ mutants, this site might be inaccessible to external divalent cations. Because the EEEE locus is thought to be situated near the extracellular entrance to the pore, a putative second high-affinity binding site might be located anywhere along most of the length of the pore.

Irrespective of the specific formulations of these two criticisms, their salience lies in the exposure of a weakness in the case for a single high-affinity locus model of selective permeability in Ca\(^{2+}\) channels. In this paper, we present two tests of the validity of these arguments: we have used the substituted-cysteine accessibility method (Akabas et al., 1992) to compare side-chain orientation in the AAAA mutant with that of the wild-type channel, and we have probed from the intracellular side of the channel for high-affinity pore binding of Ca\(^{2+}\) in channels lacking an EEEE locus (AAAA and QQQQ mutants).

**Materials and Methods**

Molecular Biology and Expression of Ca\(^{2+}\) Channels

To enhance channel expression in *Xenopus* oocytes, cDNAs for the pore-forming \(\alpha_{1C}\) (Mikami et al., 1989) and the auxiliary \(\beta_{2b}\) (Hullin et al., 1992) were subcloned into a modified version of the pGEMHE vector (modified polylinker), which contains the 5’ and 3’ untranslated regions of the *Xenopus* \(\beta\)-globin gene (Liman et al., 1992). The \(\alpha_{1C}\) cDNA was also reconstructed to include a consensus Kozak sequence for initiation of translation. Quadruple alanine (AAAA) and quadruple glutamine (QQQQ) mutants were made by substituting either alanine (A) or glutamine (Q) for the EEEE locus glutamate (E) in each of the four motifs of wild-type (WT) \(\alpha_{1C}\). All mutants were constructed using megaprimer PCR-based mutagenesis (Barik, 1995) within cassettes centered on the EEEE locus glutamate codons. Each cassette was delimited by a pair of unique restriction sites (motif I: SalI–BamHI, 308 bp; motif II: StuI–EcoRI, 380 bp; motif III: SalI–SnaBI, 401 bp; motif IV: SacII–BclI, 371 bp). The four single A or four single Q mutations were subsequently combined to make the AAAA or QQQQ constructs. For experiments in which amino acid side-chain accessibility was probed with methanethiosulfonate reagents, single cysteine (C) substitutions were introduced at various positions in the AAAA mutant using a similar PCR-based mutagenesis strategy. Mutations and cassette sequence fidelity were confirmed by cDNA sequencing.

Ovarian tissue was removed from anesthetized female *Xenopus laevis* (*Xenopus One*) and agitated in Ca\(^{2+}\)-free OR-2 solution (mM: 82.5 NaCl, 2.5 KCl, 1 MgCl\(_2\), 5 HEPES, pH 7.6 with NaOH)

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Abbreviations used in this paper: GFP, green fluorescent protein; MTSEA, 2-aminoethy methanethiosulfonate; WT, wild type.
containing 2 mg/ml collagenase A or B (Boehringer) for 60–120 min. After a 30-min rinse in fresh OR-2 solution, stage V and VI oocytes were selected by hand. cRNAs encoding $\alpha_{1C}$ (WT or mutant), $\alpha_{2b}$, and $\beta_2b$ subunits were transcribed in vitro using the mMESSAGE mMACHINE kit (Ambion) and injected into oocytes in an equimolar ratio. Injected oocytes were stored at 18°C in ND-96 solution (mM: 96 NaCl, 2 KCl, 1.8 CaCl$_2$, 1 MgCl$_2$, 5 HEPES, pH 7.6 with NaOH) containing 2.5 mM sodium pyruvate (Sigma-Aldrich), 100 U/ml penicillin (Sigma-Aldrich), 0.1 mg/ml streptomycin (Sigma-Aldrich), and 0.1 mg/ml gentamicin (Boehringer) and studied 3–14 d after injection.

For expression in HEK293 cells, WT and mutant cDNAs were subcloned into the pbK-CMV N/B-200 vector, a version of pbK-CMV (Stratagene) in which the lac promoter and the initiation codon for β-galactosidase have been removed. HEK293 cells (CRL-1573; American Type Culture Collection) were grown at 37°C and 5% CO$_2$ in Minimal Essential Medium (GIBCO BRL) supplemented with 10% fetal bovine serum (GIBCO BRL), 100 U/ml penicillin (Sigma-Aldrich), and 0.1 mg/ml streptomycin (Sigma-Aldrich). An equimolar ratio of $\alpha_{1C}$, $\alpha_{2b}$, $\beta_2b$, and green fluorescent protein (GFP; GIBCO BRL) cDNAs were transiently transfected using the Effectene transfection kit (QIAGEN). Cells expressing GFP (as determined by their fluorescence) were studied 48–72 h after transfection. Approximately 10–20% of cells were transfected (expressed GFP) under these conditions.

Electrophysiological Recording from Xenopus Oocytes

Single-channel currents were recorded from cell-attached patches on oocytes that had been stripped of their vitelline membrane. The oocytes were bathed in a high K$^+$ solution designed to zero the membrane potential (mM: 100 KCl, 10 EGTA, pH 7.4 with TEA-OH). The Ca$^{2+}$-channel agonist FPL 64176 (RBI) was included in the bath solution (2 μM) to prolong channel open time, which facilitated the study of pore block. Patch pipets were made of borosilicate glass (Warner Instruments Co.), coated with Sylgard (Dow Corning Corp.) or wax (Kerr Corp.) and had resistances of 1–3 MΩ when filled with solution for recording inward Li$^+$ currents (mM: 100 LiCl, 10 HEPES, 10 HEDTA, pH 7.4 with TEA-OH). "Chelator" software (Theo J.M. Schoenmakers, University of Nijmegen, Nijmegen, The Netherlands) was used to determine the [CaCl$_2$] for the desired free [Ca$^{2+}$]. Currents were recorded with an Axopatch 200B amplifier (Axon Instruments, Inc.). The amplifier’s internal filter was set to 10 kHz and an external filter (eight-pole Bessel filter; Frequency Devices, Inc.) was set to 2 kHz, yielding a final corner frequency of 1.96 kHz. Data were sampled at 10 kHz using Pulse software (HEKA, distributed by Instrutech Corp.).

Electrophysiological Recording from HEK293 Cells

Whole-cell currents were recorded from HEK293 cells with a 100 mM Li$^+$ solution (as above for oocytes) in the bath and a Cs$^+$ solution in the pipet (mM: 135 CsCl, 10 EGTA, 10 HEPES, 4 MgATP, pH 7.5 with TEA-OH). Patch pipets were made from thin-walled borosilicate glass (Warner Instruments Co.) and had resistances of ~1–3 MΩ. Currents were recorded and filtered with the same equipment, settings, and software as described above for single-channel current recording from oocytes, and they were sampled at 4 kHz. Leak currents were subtracted using the average of eight pulses to $-P/10$. Uncompensated series resistance was <9 MΩ, and this was compensated by 70–80% during experiments.

Following previous work by Kuo and Hess (1993a), outward Li$^+$ currents through single channels in inside-out, excised patches from HEK293 cells were recorded with 300 mM Li$^+$ in the bath (mM: 300 LiCl, 10 HEPES, 10 HEDTA, plus various [CaCl$_2$], pH 7.4 with CsOH) and 55 mM Li$^+$ in the patch pipet (mM: 55 LiCl, 55 CsCl, 10 HEPES, 10 HEDTA, 150 glucose, pH 7.4 with CsOH). CsCl$_2$ was added to the bath solution to achieve the desired free [Ca$^{2+}$], as calculated using the Chelator program. The Ca$^{2+}$-channel agonist BayK 8644 (RBI) was included in the bath solution at 5 μM to prolong channel openings; BayK 8644 was more effective than FPL 64176 at the positive potentials required to study outward currents. Pipets, amplifier, data filtering, and sampling were the same as described above for unitary current recording from the oocytes. The kinetics of pore block were analyzed using Tac and TacFit software (Bruxton). Based on the corner frequency of 1.96 kHz, the rise time of our recording system was 0.169 ms. We therefore excluded events of duration <0.2 ms from the analysis. The data were not corrected for missed events.

All chemicals for which a source is not specifically mentioned were obtained from either Sigma-Aldrich or Fluka. All mean values are reported as mean ± SEM.

**RESULTS**

Accessibility of Substituted Cysteines Suggests that AAAA Pore Structure Is Not Globally Disrupted

The fact that divalent cations do not permeate the AAAA channel raises the possibility that quadruple mutations in the EEEE locus produce a nonspecific disruption of pore structure, so that divalent cations do not permeate, although monovalent cations do. It has been suggested that this kind of disruption of structure might also destroy the postulated second high-affinity Ca$^{2+}$ binding site. As a means of assessing the structural integrity of the AAAA channel, we have compared the orientations of amino acid side-chains of pore-lining residues in WT channels with those of the corresponding residues in AAAA channels. Side-chain orientation, that is, whether a side-chain projects into the pore or not, was investigated by measuring whether a bath-applied sulfhydryl-modifying reagent could irreversibly block current through cysteine-substituted EEEE or AAAA channels. We analyzed side-chain orientation at two positions in each motif, the position occupied in...
WT channels by an EEEE locus glutamate (position 0), and the immediate amino-terminal neighbor (position –1), which is located farther from the channel’s external entrance than is the EEEE locus glutamate. For EEEE channels, four 0-position cysteine substitution mutants were studied (CEEE, ECEE, EECE, and EEEC), and, for AAAA channels, four additional 0-position mutants were studied (CAAA, ACAA, ACA, and AAAC). Cysteine substitution at the –1 position yielded four mutants in the EEEE channel (M392C, G735C, F1144C, and G1445C), and four mutants in the AAAA channel (M392C/AAAA, G735C/AAAA, F1144C/AAAA, and G1445C/AAAA).

The effects of the methanethiosulfonate reagent MT-SEA on inward, whole-cell Li⁺ currents carried by non- and cysteine-substituted AAAA channels (0 position only) are illustrated in Fig. 1 (A and B). For the non-cysteine-substituted AAAA channel, MTSEA block was small and rapidly reversible. The average steady state block of AAAA channels by MTSEA was 18 ± 1% (Fig. 2A), and the time constant for recovery from MTSEA block was 21 ± 6 s (n = 6). This unblock rate is similar to that measured for Cd²⁺ unblock in oocytes, and most likely reflects the rate at which rapidly reversible blocking agents can be washed out of the oocyte recording chamber. In contrast, the cysteine-substituted AAAA channels (CAAA, ACAA, ACA, and AAAC) were all more strongly and persistently blocked by MT-SEA.

Figure 1. Determination of side-chain orientation of pore-lining amino acids in the AAAA channel. (A) Superimposed two-electrode voltage clamp records illustrating the effects of MTSEA upon whole-cell Ca²⁺ channel currents. Currents (100 mM Li⁺) were elicited by step depolarization (150 ms) from the holding potential of –80 to –20 mV every 5 s. In each recording, after a stable baseline was established, MTSEA–Br (final concentration of MTSEA: 2 mM) was dissolved in the 100 mM Li⁺ solution and applied immediately to the oocyte via bath perfusion. (B) Time course of MTSEA action on peak inward Li⁺ currents.
SEA. Average values of MTSEA block for the 0 position cysteine mutants were 63% for CAAA, 83% for ACA, 90% for AACA, and 90% for AAAC (Fig. 2 A). All of the testable 2 position cysteine substitutions were also persistently blocked by MTSEA, with average values of block being 32% for M392C/AAAA, 52% for F1144C/AAAA, and 67% for G1445C/AAAA. The G735C/AAAA either did not express or did not produce functional channels, since oocytes injected with this cRNA exhibited little or no inward Li+ current.

The results of MTSEA block of WT EEEE channels and cysteine-substituted EEEE channels are summarized in Fig. 2 B. WT EEEE channels were blocked by only 16 ± 3%, and this small block was reversed with wash. In contrast, all −1- and 0-position cysteine substitution mutants of the EEEE channel were persistently blocked by MTSEA. For these mutants, percent block values ranged from 83 to 92% for 0-position mutants, and from 62 to 80% for −1-position mutants. These results indicate that all eight −1- and 0-position side-chains project into the pore of the EEEE channel, as has been described previously (Chen et al., 1996; Klockner et al., 1996; Chen and Tsien, 1997; Wu, X., H.D. Edwards, and W.A. Sather, manuscript submitted for publication).

Comparison of the MTSEA block patterns for EEEE and AAAA channels shows that all −1- and 0-position mutants were blocked by MTSEA in EEEE and AAAA channels, indicating that all of the tested positions are accessible to MTSEA in both channel types. Furthermore, within each motif, MTSEA block of the 0-position mutant was larger than that of the −1-position mutant for both EEEE and AAAA channels. These points of similarity in the MTSEA block patterns indicate that the orientations of amino acid side-chains at the 0 and −1 positions were not drastically altered by introduction of four alanines at the EEEE locus. In other details, the block patterns differed so that, for some cysteine substitution positions, fractional block by MTSEA differed in degree between the EEEE and AAAA channels. In comparing cysteine-substituted EEEE and AAAA channels at a given position, the differences in degree of fractional block by MTSEA are not surprising: with four small alanine side chains (−CH3) projecting into the pore in place of four larger, and charged, glutamate side chains (−CH2CH2COO−), substituted cysteine thiols may be somewhat differently accessible, or these thiols may be modified to a somewhat different degree, or thiol modification may not result in an identical degree of physical obstruction of the pore. The key finding, however, is that the EEEE and AAAA block patterns exhibited a degree of similarity that suggests that structural differences between the AAAA channel and WT are largely limited to the side-chain makeup of the EEEE locus.

**High-affinity Block by External Ca2+ Occurs at the EEEE Locus**

EEEE locus mutations have previously been shown to attenuate block by divalent cations entering the pore via its extracellularly oriented, or external, entrance (Kim et al., 1993; Tang et al., 1993; Yang et al., 1993; Yatani et al., 1994; Ellinor et al., 1995; Parent and Go-
The IC_{50} for external Ca^{2+} block of whole-cell, inward Li^{+} currents in WT α_{1C} (\sim 1 \mu M) was shifted \sim 10–50-fold by single alanine substitutions and \sim 1,000-fold by substitution of alanine for all four EEEE locus glutamates (AAA; Ellinor et al., 1995). Here we have studied effects of the EEEE locus quadruple mutations upon various configurations of Ca^{2+} block of single-channel Li^{+} currents, starting with block by external Ca^{2+} of inward Li^{+} current. In cell-attached patches on oocytes expressing WT α_{1C} channels, step depolarizations in the presence of 2 \mu M FPL 64176 produced inward Li^{+} currents of long duration (Fig. 3 A, top). Including 3 \mu M Ca^{2+} in the 100-mM Li^{+} solution bathing the extracellular surface of the patch (pipet solution) introduced frequent interruptions in the unitary inward currents, which represent individual Ca^{2+} block/unblock transitions. By contrast, inward Li^{+} currents through AAAA mutant channels were not detectably affected by \sim 3 \mu M, or even 100 \mu M, external Ca^{2+} (Fig. 3 A, bottom). When 1 mM Ca^{2+} was included in the pipet pipet solution, a small degree of flicker block was observed. This presumably is the manifestation of Ca^{2+} binding to a low affinity site in the pore. These results coincide with those obtained at the whole-cell level (Yang et al., 1993; Ellinor et al., 1995), demonstrating that high-affinity binding of divalent cations that enter the pore from the external solution occurs at the EEEE locus; no high-affinity binding is detectable in the AAAA mutant channel when probed by ions entering from the external solution.

Examples of the high fluxes of both divalent (Ba^{2+}) and monovalent (Li^{+}) cations through WT α_{1C} channels are illustrated in Fig. 3 B (left). Fig. 3 B (right) shows that, although Li^{+} is highly permeant in the AAAA channel, Ba^{2+} is not. In fact, lack of divalent cation permeability is characteristic of quadruple EEEE locus mutants, so that little or no inward current was observed with external Ba^{2+} or Ca^{2+} in the AAAA or QQQQ mutants (Ellinor et al., 1995). The immeasurably low permeability of quadruple EEEE locus mutants to divalent cations raises a key question for the one-site model of Ca^{2+} channel pore function: is it possible that another high-affinity site exists, distinct from the EEEE locus and deeper in the pore, and that this deeper site is not accessible to external divalent cations in the quadruple EEEE locus mutants?

Outward Ca^{2+} Channel Currents in Excised Patches

If there is a second high-affinity binding site for divalent cations located further from the external pore entrance than is the EEEE locus, then this deeper site could reasonably be expected to be accessible from the intracellular side of WT, AAAA, and QQQQ channels. We addressed this possibility by studying, in Ca^{2+} channel-bearing inside-out patches, block of outward Li^{+} currents by Ca^{2+} entering the pore from the internal (cytosolic) pore entrance. For this set of experiments, we had greater success studying Ca^{2+} channels expressed in HEK293 cells than in oocytes. In inside-out patches from oocytes, with 100 mM Li^{+} in the bath (internal solution), outward single-channel Li^{+} currents were not readily observed. L-type Ca^{2+} channels are known to exhibit rapid rundown or loss of activity after patch excision, perhaps due to the loss of positive regulation by intracellular components (Armstrong and Eckert, 1987; Lambert and Feltz, 1995; Hao et al., 1998). In addition, single-channel outward currents through Ca^{2+} channels in either cell-attached or excised patches have been notoriously difficult to study, possibly due to block by intracellular Mg^{2+} (Kuo and Hess, 1993c) and/or polyamines (Sjöholm et al., 1993; Gomez and Hellstrand, 1995). Xenopus oocytes in particular have high concentrations of internal polyamines that do not easily wash out even from excised patches and that hamper the observation of unitary outward currents carried by K^{+} channels (Lu et al., 1999). Use of HEK293 cells, addition of HEDTA to the bath for chelation of divalent cations including Mg^{2+}, and a higher concentration (300 mM) of Li^{+} in the bath facilitated the observation of unitary outward Li^{+} currents in inside-out excised patches. Nevertheless, channel activity was observed in only \sim 12–15% of excised patches. Rundown, as well as residual block by patch-associated polyamines and Mg^{2+}, was likely responsible for this low probability of observing unitary outward currents.

Five lines of evidence indicate that the single-channel currents that we recorded from inside-out patches were produced by transfected Ca^{2+} channels and not by ion channels endogenous to HEK293 cells. This is a particularly significant issue for the AAAA and QQQQ mutant channels, since their unitary conductance and block properties have not been previously described. First, consider the endogenous channels of HEK293 cells. Although Ca^{2+} channels native to HEK293 cells display some of the characteristics of L-type Ca^{2+} channels, these native channels are insensitive to the dihydropyridine agonist BayK 8644, and they were therefore not responsible for the long-duration openings that we studied (Berjukow et al., 1996). The activity of K^{+} channels native to HEK293 cells is sparse, their conductances are inconsistent with those we have measured (Yu and Kerchner, 1998; Zhu et al., 1998), and K^{+} channels are typically only poorly permeated by Li^{+}, with P_{Li}/P_{K} permeability ratios generally <0.1 (Hille, 1992). The unitary conductances of the three Cl^{-} channels identified in HEK293 cells are (in symmetrical 150 mM Cl^{-}) 55, 240, and 350 pS (Zhu et al., 1998). These large conductances are expected to be even larger with the 300 mM internal and 260 mM external Cl^{-} used in our experiments, and such large conductances differ...
greatly from those we have investigated. Cl\(^-\) channels in HEK293 cells are also relatively rare, occurring in only 4–5% of patches (Zhu et al., 1998).

Second, reversal potentials determined from whole-cell current–voltage relationships were consistent with the overwhelming majority of current in transfected cells being attributable to exogenous Ca\(^{2+}\) channels (Fig. 4 A). Average reversal potential for \(\alpha_{1C}\)-transfected cells was +31.2 ± 2.2 mV (n = 5), reflecting a significant preference in the WT \(\alpha_{1C}\) channel for Li\(^+\) over Cs\(^+\). AAAA-transfected cells had a reduced preference for Li\(^+\) over Cs\(^+\) (average reversal potential = +10.7 ± 1.3 mV, n = 6), which is consistent with the loss of selectivity caused by the quadruple EEEE locus mutations.
Third, measurement of whole-cell currents in HEK293 cells demonstrated that the Li\(^+\) currents through single channels in excised patches were dominantly due to permeation of heterologously expressed Ca\(^{2+}\) channels, rather than endogenous channels. Fig. 4 B shows that inward Li\(^+\) current density was \(\sim\)50-fold higher for cells expressing WT \(\alpha_{1C}\) compared with control cells (GFP-transfected only) and \(\sim\)8-fold higher for cells expressing AAAA compared with control. Likewise, outward Cs\(^+\) current densities were significantly greater in Ca\(^{2+}\) channel-transfected than in control cells (\(\sim\)17-fold for WT and \(\sim\)7-fold for AAAA; Fig. 4 B).

Fourth, a dihydropyridine antagonist of L-type Ca\(^{2+}\) channels, nimodipine (10 \(\mu\)M), blocked inward Li\(^+\) currents through AAAA-transfected cells by 67 \(\pm\) 8% (\(n = 3;\ -20 \text{ mV}\)), which is similar to the degree of block of WT \(\alpha_{1C}\) channels expressed in oocytes (Furukawa et al., 1999). Fifth, and finally, in patches from \(\alpha_{1C}\)-transfected cells, when micromolar Ca\(^{2+}\) was present in the bath, WT \(\alpha_{1C}\) channels were always blocked by Ca\(^{2+}\); there was no contamination with nonblocked channels. In the case of the AAAA-transfected cells, the absence of internal Ca\(^{2+}\) block of outward Li\(^+\) currents eliminates the possibility that currents recorded from these cells might have been carried by endogenous Ca\(^{2+}\) channels.

Internal Ca\(^{2+}\) Blocks Wild-Type Ca\(^{2+}\) Channels with High Affinity

Recordings of outward Li\(^+\) currents through single \(\alpha_{1C}\) channels in inside-out patches from HEK293 cells are shown in Fig. 5 A. These channels exhibited multiple conductance states, a well-documented behavior of voltage-gated Ca\(^{2+}\) channels (e.g., Clues and Sather, 2000). The amplitude to which they opened most frequently was the largest one, and this amplitude was analyzed for kinetics of pore block. WT \(\alpha_{1C}\) channels were blocked when at least micromolar Ca\(^{2+}\) was present in the solution bathing the cytosolic surface of the patch. Representative WT single-channel records illustrate the flicker block that was introduced by internal Ca\(^{2+}\), with the number of flicker block events increasing with [Ca\(^{2+}\)] (Fig. 5 A). Quantitation of open and shut times yielded Ca\(^{2+}\) on rates (1/\(\tau_{\text{open}}\)) that increased with [Ca\(^{2+}\)] and off rates (1/\(\tau_{\text{shut}}\)) that were concentration independent, findings that are indicative of a bimolec-
ular reaction. At a membrane potential of +20 mV, the average off rate was 2,965 s⁻¹ and the on-rate coefficient, determined as the slope of a linear regression fit, was $2.3 \times 10^8$ M⁻¹ s⁻¹. The WT channel exhibited high-affinity binding of Ca²⁺ in this configuration, as the apparent $K_d$ is calculated to be $\sim 13$ μM ($k_{off}/k_{on}$). The concentration-dependent on rate is similar to that of Kuo and Hess (1993a), who studied Ca²⁺ block of Li⁺ current through endogenous Ca²⁺ channels in PC12 cells. Likewise, the magnitude and concentration independence of the off rate agree with that measured by Kuo and Hess (1993a). The voltage dependence of
block by Ca\(^{2+}\) was determined over the voltage range 0 to +20 mV. The on rate was voltage independent, whereas the off rate increased with depolarization, e-fold/19 mV (Fig. 5 B). These characteristics match those of the on and off rates obtained by Kuo and Hess (1993a), as expected if the channels we have studied are WT \(\alpha_{1c}\) channels.

AAA and QQQQ Channels Are Not Effectively Blocked by Internal Ca\(^{2+}\)

In contrast to the case for WT channels, unitary outward Li\(^{+}\) currents through AAAA channels did not exhibit detectable flicker block with internal (bath) [Ca\(^{2+}\)] as high as 1 mM (Fig. 6), nor even 3 mM (data not shown). If Ca\(^{2+}\) block and unblock of AAAA channels occurred on a time scale shorter than the dead time of our recording system, then discrete Ca\(^{2+}\) block events would not be fully resolved and Ca\(^{2+}\) block would reduce unitary current amplitude in a manner graded with [Ca\(^{2+}\)]. A potentially complicating factor is that, like WT, the AAAA mutant exhibited four clear conductance levels. However, no graded reductions in AAAA channel unitary current amplitudes were detected as internal Ca\(^{2+}\) was increased, so that AAAA channels exhibited the same unitary current amplitudes in the presence of 1 mM internal Ca\(^{2+}\) as in control internal solution. Thus, measurements of unitary current amplitudes also did not reveal block by internal Ca\(^{2+}\) of outward Li\(^{+}\) currents in AAAA channels.

QQQQ is another EEEE locus quadruple mutant that lacks high-affinity binding of Ca\(^{2+}\) when probed via the pore's external entrance (Ellinor et al., 1995). QQQQ channels behaved like AAAA channels in the present study: with 1 mM Ca\(^{2+}\) in the internal solution (bath), there was no detectable block of outward Li\(^{+}\) currents (Fig. 7). As found for the AAAA mutant, reduced unitary conductance as a consequence of fast, unresolved flicker block by Ca\(^{2+}\) was not detected for the QQQQ mutant either. The combined results from WT, AAAA, and QQQQ channels argue that Ca\(^{2+}\) entering the channel from the intracellular entrance can bind with high affinity, but only in channels with an intact EEEE locus, and that in the absence of an intact EEEE locus, there is no high-affinity binding and therefore no second high-affinity binding site in the pore.

**DISCUSSION**

Side-Chain Orientation in the AAAA Mutant

Because high-resolution structures are not available for the pores of mutant or WT Ca\(^{2+}\) channels, the effects of mutations upon pore structure can only be inferred from indirect information such as altered pore block. This is one reason why the idea that Ca\(^{2+}\) channels possess only a single locus of high-affinity binding in their pore-lining regions has remained tantalizingly short of conclusive.

We have tried to determine whether amino acid substitutions in the EEEE locus have little effect on structure other than to replace one side chain with another while preserving general spatial organization, or whether substitutions may have in addition the undesired effect of significantly altering side-chain orientation: in one extreme example, side-chains that project into the lumen of the WT pore to interact with permeating ions might, upon amino acid substitution, project away from the lumen of the mutant pore. Even more problematic, it is possible that EEEE locus substitutions could conceivably cause significant reorientation of side chains outside the EEEE locus. To address this kind of prob-
Figure 7. Outward Li$^+$ currents through QQQQ channels are not blocked by internal Ca$^{2+}$. Currents were studied in inside-out patches excised from HEK293 cells expressing QQQQ channels. The bath contained 300 mM Li$^+$ plus either 3 nM Ca$^{2+}$ (control) or 1 mM Ca$^{2+}$, and the patch pipet contained 55 mM Li$^+$. Single-channel currents were recorded during a test depolarization to +20 mV, following a 25- or 50-ms prepulse to +100 mV. Holding potential was −100 mV.

Problem, we investigated the accessibility of substituted cysteines to determine side-chain orientation in the pore, but one view of this approach is that relying on additional mutagenesis work in the absence of "real" structural information only compounds the problems inherent in the earlier mutagenesis work. This may be true. Yet atomic-scale pore structures for both WT and mutant Ca$^{2+}$ channels are not likely to be in hand any time soon, and further mutagenesis work allows comparison of the effects of various mutations, thereby providing tests for the consistency of conclusions regarding pore structure. Following this logic, we have examined whether side chains that have been found in previous mutagenesis work to project into the pore of WT channels also do so in the relatively severe AAAA mutant.

Based on substituted-cysteine accessibility analysis of WT channels (Wu, X., H.D. Edwards, and W.A. Sather, manuscript submitted for publication) and on evidence that the EEEE locus side-chains together form a single protonatable site in the pore (Chen et al., 1996; Klockner et al., 1996; Chen and Tsien, 1997), in WT EEEE channels the side chains at the 0 and −1 positions project into the pore. Cysteines substituted at three of the four −2 positions in the EEEE channel did not support MTSEA block, leaving the side-chain orientation at these positions unknown for now (Wu, X., H.D. Edwards, and W.A. Sather, manuscript submitted for publication). Here, we have found that cysteines substituted into the AAAA mutant at all 0 and all test—1 positions are susceptible to long-lasting modification by MTSEA. By this very simple index, side-chain orientation thus appears to be at least crudely preserved despite the introduction of four or more mutations into the selectivity filter region.

Because we were specifically concerned about the possible existence of Ca$^{2+}$ binding sites located internally to the EEEE locus, we did not examine side-chain orientation at more external positions (+1, +2) in the AAAA channel. Examination of substituted-cysteine accessibility at deeper pore positions was not attempted, based on suspected large-scale structural similarity with the KcsA pore structure (Doyle et al., 1998). Only a short segment of the P-loop in the KcsA K$^+$ channel lines the pore and analysis of protein secondary structure for Ca$^{2+}$ channels strongly suggests that the P-loops of these channels have a roughly similar architecture to that of the KcsA channel. Specifically, a pore helix, which does not line the pore in KcsA channels, is predicted to occupy positions −4 through −15 in all Ca$^{2+}$ channel P-loops. Thus, at this point, residues lining the deeper regions of the Ca$^{2+}$ channel pore remain largely unknown. Furthermore, the deeper pore of Ca$^{2+}$ channels is very likely to comprise an aqueous cavity like that found in the KcsA channel, because this aqueous cavity is thought to be essential in lowering the dielectric barrier to ion permeation through cell membranes. Though Ca$^{2+}$ occupancy of this region of the pore may be important in supporting high Ca$^{2+}$ flux, it is difficult to envision how a high-affinity Ca$^{2+}$ binding site in such a wide-diameter section of the pore could be involved in the selectivity-by-binding process.

Considering all of the above, the most probable conclusion that can be drawn from the substituted-cysteine accessibility work is this: despite the limited nature of the information provided, the concern that fourfold alanine substitution at the EEEE locus might result in substantial rearrangement of pore structure seems unlikely owing to the absence of a drastic affect on structure near and at the EEEE locus. Even so, a viable alternative view is that the small structural differences detected between EEEE and AAAA channels hint at larger structural differences deeper in the pore. Identification of sequences lining the deeper pore will allow the more extensive comparison of pore structure between EEEE and AAAA channels needed to address this lingering concern.

In contrast to the situation with cysteine-substituted mutants, the modest block by MTSEA of the non-cysteine-substituted AAAA channel was reversible, and occurred at a rate similar to that of Cd$^{2+}$ unblock of Ca$^{2+}$ channels in oocytes. One reasonable interpretation of these observations is that the cationic MTSEA is attracted into the pore, where it obstructs permeant ion flow. Whatever the mechanism of this kind of block by
MTSEA, however, block did not result from covalent attachment of \(-\text{SCH}_2\text{CH}_2\text{NH}_3^+\) to the non-cysteine-substituted AAAA channel, and so this kind of block was separable from that measured for the cysteine-substituted mutants.

The Number of High-Affinity Binding Sites Involved in Selective Ion Permeability

Our examination of AAAA and QQQQ channels for block of outward \(\text{Li}^+\) current by \(\text{Ca}^{2+}\) entering the pore via its intracellularly oriented entrance showed that \(\text{Ca}^{2+}\) at up to 3 mM failed to effectively block these mutants. Specifically, we were unable to detect the kind of discrete \(\text{Ca}^{2+}\) block and unblock events that are the kinetic signature of high-affinity \(\text{Ca}^{2+}\) binding. Thus, \(\text{Ca}^{2+}\) channels lacking an intact EEEE locus are unable to bind \(\text{Ca}^{2+}\) with approximately micromolar affinity, whether \(\text{Ca}^{2+}\) enters the pore via the external entrance, as shown in previous work, or via the internal entrance, as shown here. Combining these results with substituted-cysteine accessibility results suggesting that pore structure was not extensively disrupted in the AAAA mutant solidifies the view that the EEEE locus comprises the only high-affinity \(\text{Ca}^{2+}\) binding site in the pore of voltage-gated \(\text{Ca}^{2+}\) channels. Our results are consonant with those obtained in studies of double alanine substitution mutants, which are permeated by at least one divalent cation, \(\text{Ba}^{2+}\), but that exhibit greatly weakened \(\text{Ca}^{2+}\) block and binding (Ellinor et al., 1995). As it is likely that double alanine substitutions would disrupt structure even less than would quadruple substitutions, and because the \(\text{Ba}^{2+}\) permeability of the double mutants suggests that \(\text{Ca}^{2+}\) would be able to reach and bind to any nondisrupted second site if it existed in these mutants, the lack of high-affinity \(\text{Ca}^{2+}\) binding observed in the double alanine mutants represents strong evidence against the existence of a second pore-localized, high-affinity \(\text{Ca}^{2+}\) binding site.

In the AAAA and QQQQ mutants, 1 mM internal \(\text{Ca}^{2+}\) not only failed to produce resolvable, discrete block/unblock events, but this very high level of \(\text{Ca}^{2+}\) also failed to produce a detectable change in unitary \(\text{Li}^+\) current amplitude. Two-electrode voltage-clamp measurements in oocytes have shown that currents carried by 100 mM \(\text{Li}^+\) through AAAA and QQQQ channels are half-blocked by \(\sim 1.2\) mM \(\text{Ca}^{2+}\) (Ellinor et al., 1995), so it might have been anticipated that AAAA or QQQQ unitary current amplitudes would be reduced by \(\sim 50\%\) at 1 mM \(\text{Ca}^{2+}\), despite the absence of any high-affinity \(\text{Ca}^{2+}\) binding site. However, the recording conditions and solutions differ between two-electrode voltage-clamp and inside-out patch work, shifting the half-block value for WT from the 1-\(\mu\)M value measured by two-electrode voltage clamp to 13 \(\mu\)M, measured here with inside-out patches. Scaling the mutant channel half-block values by this factor of 13 predicts that mutant channels would only be blocked by \(6\%\) at 1 mM \(\text{Ca}^{2+}\), a reduction in unitary current amplitude so small that we were unable to detect it.

An External Low-Affinity \(\text{Ca}^{2+}\) Binding Site?

We were unable to detect discrete block events in AAAA channels when \(\text{Ca}^{2+}\) entered the pore from the internal entrance, but we did detect what appeared to be discrete block events when \(\text{Ca}^{2+}\) entered the AAAA pore via the external entrance. Although it was difficult to separate the external block events from channel gating events, there appeared to be more resolved open/shut transitions in high \(\text{Ca}^{2+}\) than in low \(\text{Ca}^{2+}\) (Fig. 3 A). Assuming that the brief open/shut transitions produced in high external \(\text{Ca}^{2+}\) represent block of \(\text{Li}^+\) flux by \(\text{Ca}^{2+}\) binding in the permeation pathway, where could such binding occur in AAAA channels that lack an EEEE locus? A speculative possibility is that \(\text{Ca}^{2+}\) binds to a low-affinity site just external to the EEEE locus. Such a site would necessarily possess low \(\text{Ca}^{2+}\) binding affinity because the AAAA channel is only weakly blocked by \(\text{Ca}^{2+}\); an external localization is plausible based on the fact that \(\text{Ca}^{2+}\), which is impermeant in the AAAA channel, produced discrete block only when present on the external side of the membrane. Detection of discrete block events is possible for a low-affinity site assuming that a large majority of block events are too brief to be resolved, and that only the rare, very longest events in the distribution of blocked-state lifetimes are detected.

The low-affinity block by \(\text{Ca}^{2+}\) of monovalent cation flux through AAAA channels (IC_{50} \sim 1\ mM) has been thought, since it was first observed, to be mediated by a superficial, externally localized site (Ellinor et al., 1995). WT \(\text{Ca}^{2+}\) channels also possess a low-affinity, superficial metal ion binding site (Polo-Parada and Korn, 1997). Perhaps these previously considered external, low-affinity \(\text{Ca}^{2+}\) binding sites are one and the same with the low-affinity site we have speculated upon here, a site that may represent the physical correlate of the external flanking potential energy well in the Dang and McCleskey (1998) step model of selective permeability in \(\text{Ca}^{2+}\) channels.

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