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# Structural determinants of ion selectivity in brain calcium channel

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Glutamic acid residues in the SS2 segment of the internal repeats III and IV of the brain calcium channel BI were subjected to single point mutations. The mutant channels were tested for macroscopic current properties and sensitivities to inorganic blockers. The mutation that replaces glutamic acid 1,469 with glutamine altered ion-selection properties and strongly reduced the sensitivity to $Cd^{2+}$, whereas the analogous mutation of glutamic acid 1,765 exerted smaller effects on ion-selection properties. Our results indicate that these glutamic acid residues, equivalently positioned in the aligned sequences, play different roles in the selective permeability of the calcium channel.

Calcium channel; Site-directed mutagenesis; cDNA expression; Ion selectivity; Inorganic blocker

## 1. INTRODUCTION

Voltage-dependent calcium channels control the entry of $Ca^{2+}$ across the surface membrane of excitable cells and thereby influence electrical activity and diverse cellular responses. Under physiological conditions, calcium channels must be highly selective in allowing large $Ca^{2+}$ fluxes to the exclusion of other ions such as $Na^+$, $K^+$ and $Mg^{2+}$, even though these ions are present at comparatively higher concentrations. Although several theoretical models have been proposed to explain this selective permeability [1–4], little direct information concerning its structural basis has been available [5]. Recently, amino acid substitutions in the SS1–SS2 region of potassium channels and sodium channels have been shown to alter their ion selectivity and/or permeability [6–14]. In addition, the region is also important for sensitivities to pore blocking agents [6,8,11,12,15–19]. It is therefore possible that the SS1–SS2 region of calcium channels is also involved in the formation of the pore structure. The present investigation deals with the effects on ion permeation and sensitivity to inorganic blockers of site-directed mutations in the SS2 segment of the brain calcium channel BI. The results obtained reveal that glutamic acid residues 1,469 and 1,765 located in repeat III and repeat IV, respectively, are involved differently in determining ion selectivity of the brain calcium channel.

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## 2. MATERIALS AND METHODS

The recombinant plasmid pSPCBI-2 [20], which carries the entire protein-coding sequence of the rabbit brain calcium channel BI cDNA linked with the bacteriophage SP6 promotor [21], and its mutants were used for synthesis in vitro of specific mRNAs. The plasmids carrying mutants were constructed [22], using oligodeoxyribonucleotides prepared with an automatic DNA synthesizer (Applied Biosystems). The mutant plasmids differ from pSPCBI-2 as follows (the substituted nucleotides with residue numbers [20] are given and the plasmids carrying mutant cDNAs are named after the mutant specification). pBI(E1469Q): C, 4405. pBI(E1765Q): C, 5293. mRNAs specific for the wild-type and mutant calcium channels, skeletal muscle $\alpha_2$ and $\beta$ subunits were synthesized in vitro [21], using *Xba*I-cleaved pSPCBI-2 and its mutants, *Sal*I-cleaved pSPCA1 [23] and *Xba*I-cleaved pCaB1 [20], respectively, as templates.

*Xenopus laevis* oocytes were injected with either the wild-type or a mutant BI-2-specific mRNA (0.3 $\mu g/\mu l$) in combination with the $\alpha_2$ subunit-specific mRNA (0.3 $\mu g/\mu l$) and the $\beta$ subunit-specific mRNA (0.1 $\mu g/\mu l$) and incubated as described in [20]. Whole-cell currents were recorded with a two-microelectrode voltage clamp at 20 ± 2°C. The external bathing solution had the following composition (in mM): 40 $Ba^{2+}$, 50 $Na^+$, 2 $K^+$ (pH 7.4 with methanesulphonic acid) [24]. Current records were sampled at 0.4-ms intervals after low-pass filtering at 0.3 kHz. The current signals have been corrected for leakage and capacitive transients using a P/4 procedure. Inorganic blocker sensitivity was assayed by perfusing $Cd^{2+}$, $Ni^{2+}$, $Co^{2+}$ and $La^{3+}$-containing external solutions.

## 3. RESULTS

Fig. 1 shows an alignment of the amino-acid sequences in the regions encompassing the short segment SS2 of the four repeats of calcium channels [20,23,25–27] (and Fujita et al., in preparation) and the sodium channel II [28]. Negatively charged amino acid residues

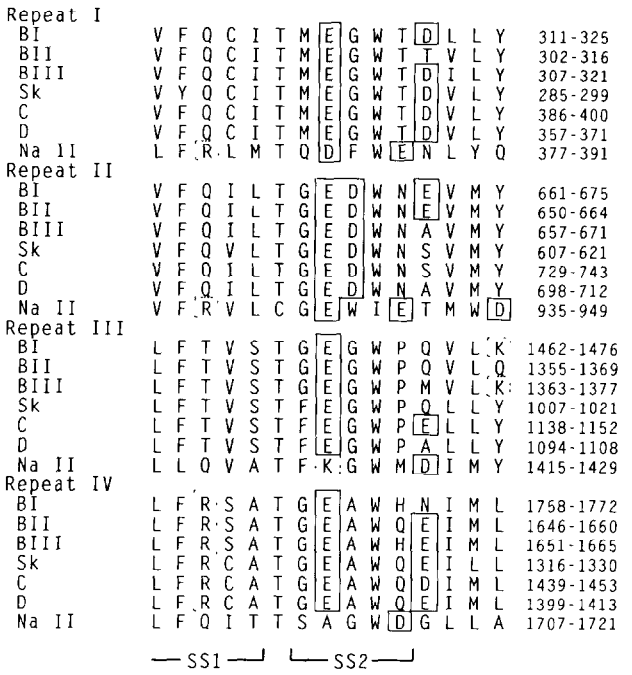


Fig. 1. Alignment of the amino-acid sequences (in single-letter code) in the regions encompassing the SS2 segment of the four repeats of different calcium channels and rat brain sodium channel II. The sequences are as follows: rabbit brain calcium channel BI (BI; [20]), rabbit brain calcium channel BII (BII; [25]); rabbit brain calcium channel BIII (BIII; Fujita et al., in preparation); rabbit skeletal muscle dihydropyridine (DHP)-sensitive calcium channel (Sk; [26]); rabbit cardiac DHP-sensitive calcium channel (C; [23]); human DHP-sensitive calcium channel $\alpha_{1D}$ (D; [27]); rat brain sodium channel II (Na II; [28]). The numbers of the amino-acid residues in each sequence are given on the right-hand side. The positions of the SS1 and SS2 segments [38] are indicated at the bottom. Negatively charged residues are boxed with solid lines, and positively charged residues with broken lines.

(Glu) of this segment in the four internal repeats of the calcium channels can be aligned at the equivalent position to form a cluster. The corresponding positions of the sodium channel are occupied by negatively charged residues in repeats I and II (Asp and Glu, respectively), but by a positively charged residue (Lys) in repeat III and an uncharged residue (Ala) in repeat IV. Substitutions of Glu for Lys in repeat III and for Ala in repeat IV of the sodium channel conferred ion permeation properties of calcium channels on the sodium channel [13]. This result led to the idea that Glu residues in the segment SS2 of repeats III and IV are important in determining ion selectivity of the calcium channels. We therefore examined ion permeation properties of the mutated calcium channels in which a glutamine residue is substituted for Glu-1,469 (E1469Q) or for Glu-1,765 (E1765Q).

Fig. 2 shows whole-cell current records from *Xenopus* oocytes injected with mRNA specific for the wild-type BI calcium channel (Fig. 2A), the mutant E1469Q (Fig. 2B) or the mutant E1765Q (Fig. 2C), measured with high external $Ba^{2+}$ concentration [24]. A major difference between the wild type and the mutant channels is the larger outward currents relative to the inward currents in the mutants. The current–voltage relationships of the wild type and the mutants allow a more detailed comparison (Fig. 2 lower panels). The reversal potential for the mutant E1469Q was markedly shifted to the negative direction (+37.5 ± 2.9 mV; mean ± S.D., $n$ = 12, $n$ indicating the number of oocytes), compared to that for the wild type (+57.6 ± 3.9 mV; $n$ = 6). Although smaller in degree, the mutation E1765Q also caused a shift in reversal potential to the negative direction (+49.8 ± 3.0 mV; $n$=11). These results suggest that two mutations altered the strict selectivity for divalent cations over monovalent cations, which is a defining attribute of the calcium channels [29,30].

To further characterize the structural alteration caused by mutations, we studied blocking effects of various inorganic cations $Cd^{2+}$, $Ni^{2+}$, $Co^{2+}$ and $La^{3+}$. Fig. 3 shows dose–response curves for blockage of inward currents by polyvalent metal ions for the wild type and the mutants E1469Q and E1765Q. While the $IC_{50}$ of $Cd^{2+}$ (concentration required for inhibiting peak current by 50%) was considerably low for the wild type, the mutation E1469Q made the $IC_{50}$ value for $Cd^{2+}$ more than 200 times larger. This mutation had an insignificant effect on the sensitivity to $Co^{2+}$ or $Ni^{2+}$, but marginally decreased the sensitivity to $La^{3+}$. The mutation E1765Q produced minor or insignificant changes in sensitivity to inorganic cations.

## 4. DISCUSSION

In the present investigation, we have shown that the mutations substituting Gln residues for Glu in the segment SS2 of repeats III and IV alter ion-permeation properties of the brain calcium channel. The shift in reveral potential of whole-cell current to the negative direction, more prominent for the mutant E1469Q than the mutant E1765Q, indicates that the mutations altered the strict permeating selectivity for divalent cations over monovalent cations, and implies that these Glu residues differently take part in forming the selectivity filter of the calcium channel.

We have also investigated effects of the mutations on sensitivities of the whole-cell currents to inorganic polyvalent cations, because it is generally accepted that many of them including the transition metals $Ni^{2+}$, $Cd^{2+}$, $Co^{2+}$ and $Mn^{2+}$ block the calcium channels by competing for common binding sites with permeant cations $Ca^{2+}$, $Ba^{2+}$ or $Sr^{2+}$ [31–33]. Furthermore, a marked sensitivity to $Cd^{2+}$ is one of the important criteria to distinguish neuronal high voltage-activated (HVA) calcium channels from a low voltage-activated (LVA) calcium channel, which is highly sensitive to $Ni^{2+}$ [34,35]. In our present study, the mutation E1469Q in repeat III dramatically decreased sensitivity to $Cd^{2+}$, without af-

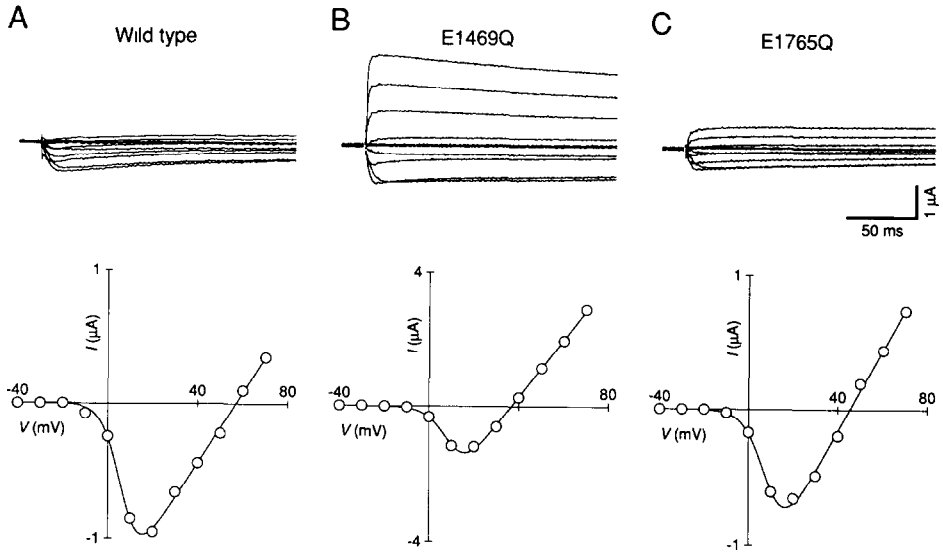


Fig. 2. Whole-cell currents recorded from *Xenopus laevis* oocytes expressing the wild-type BI calcium channel (A), the mutant E1469Q (B) and the mutant E1765Q (C). Upper panel: current traces in response to depolarizing pulses ranging from −40 to +70 mV in steps of 10 mV from a holding potential of −80 mV. Lower panel: peak current–voltage relationships. Continuous lines represent the non-linear least square fits according to $y = G_{max} \times (x - V_{rev})/(1 + \exp((V^m_{1/2} - x)/a_m))$. The values of $V_{rev}$ are +56.9 mV for the wild type, +36.7 mV for E1469Q and +45.3 mV for E1765Q. No detectable current was observed for non-injected oocytes or oocytes injected with the $\alpha_2$ and $\beta$ subunit-specific mRNA.

fecting sensitivities to other inorganic blockers very much. The mutation E1765Q in repeat IV did not significantly change sensitivities to inorganic blockers including $Cd^{2+}$. These results indicate that Glu-1,469 in repeat III is essential in forming a structural motif specific for the HVA channel pore.

Although Glu-1,469 in repeat III and Glu-1,765 in repeat IV are positioned equivalently in the aligned sequence, our present results have demonstrated functional differences between the two residues, indicating that the Glu residues in the four repeats are unlikely arranged in a tetrad-symmetric fashion around the cen-

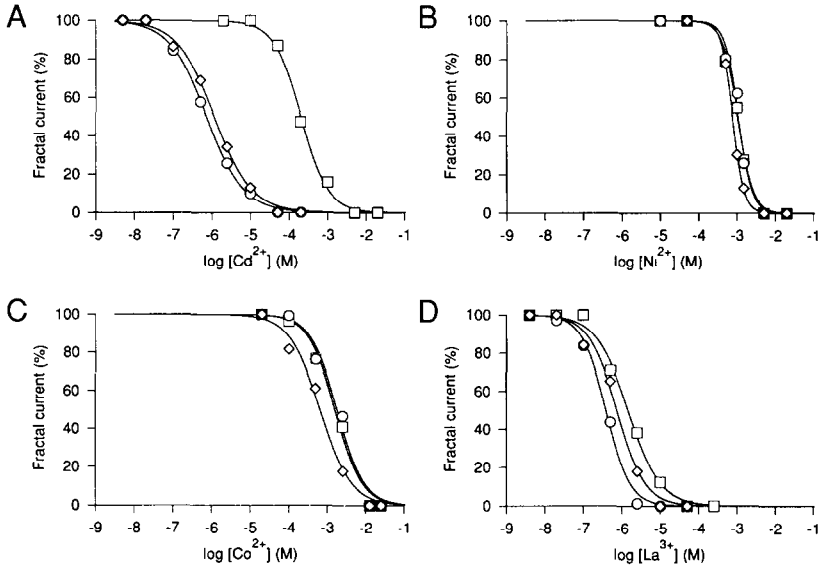


Fig. 3. Blocking effects of $Cd^{2+}$ (A), $Ni^{2+}$ (B), $Co^{2+}$ (C) and $La^{3+}$ (D) on whole-cell currents from oocytes expressing the wild-type BI calcium channel (circles), the mutant E1469Q (squares) or the mutant E1765Q (diamonds). Averaged fractional peak-inward currents from 5–7 experiments were plotted as a function of external concentration of test ions. Currents were evoked by a step from −80 mV to +20 mV. Continuous lines represent the non-linear least square fits according to $y = 100/(1 + (x/IC_{50})^n)$. The values of $IC_{50}$ and a Hill coefficient ($n$) are as follows. $Cd^{2+}$: 0.73 $\mu$M and 0.90 for the wild type, 201 $\mu$M and 1.23 for E1469Q and 1.16 $\mu$M and 0.89 for E1765Q. $Ni^{2+}$: 1.08 mM and 2.46 for the wild type, 1.04 mM and 2.18 for E1469Q and 0.76 mM and 2.93 for E1765Q. $Co^{2+}$: 1.79 mM and 1.24 for the wild type, 1.57 mM and 1.24 for E1469Q and 0.65 mM and 1.08 for E1765Q. $La^{3+}$: 0.38 $\mu$M and 1.39 for the wild type, 1.45 $\mu$M and 1.03 for E1469Q and 0.74 $\mu$M and 1.18 for E1765Q.

tral axis of the pore of the channel. This deviation from the symmetry may partly be attributable to the positions of adjacent glycine residues (Gly) relative to the Glu residues. Through all the calcium channels, Gly residues are conserved on the C-terminal side of Glu-318 in repeat I and Glu-1,469 in repeat III, and on the N-terminal side of Glu 668 in repeat II and Glu-1,765 in repeat IV. Furthermore, an additional Gly is present on the N-terminal side of the Glu in repeat III of the brain channels. This may confer a wide conformational space and flexibility on the polypeptide chain to make Glu in repeat III more accessible to ions passing through the channel pore than Glu in repeat IV. It is worth mentioning that amino-acid sequences similar to the SS2 segment in repeats I (**TMEGW**) and III (**TGEGW**) are found at the $Ca^{2+}$-binding domain in the crystal structures of troponin C from chicken skeletal muscle and turkey skeletal muscle (**NADGF**) [36,37]. The aspartic acid residue in this turn structure constitutes one of the coordination ligands of $Ca^{2+}$ in loop III. This finding supports our notion that Glu residues in the SS2 segment are important determinants of ion selectivity in the calcium channels.

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