Pore properties and ionic block of the rabbit epithelial calcium channel expressed in HEK 293 cells

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1. We have used the whole-cell patch-clamp technique to analyse the permeation properties and ionic block of the epithelial Ca$^{2+}$ channel ECaC heterologously expressed in human embryonic kidney (HEK) 293 cells.

2. Cells dialysed with 10 mM BAPTA and exposed to Ca$^{2+}$-containing, monovalent cation-free solutions displayed large inwardly rectifying currents. Their reversal potential depended on the extracellular Ca$^{2+}$ concentration, [Ca$^{2+}$]o. The slope of the relationship between reversal potential and [Ca$^{2+}$]o on a logarithmic scale was $21 \pm 4$ mV, compared with $29$ mV as predicted by the Nernst equation ($n = 3-5$ cells).

3. Currents in mixtures of Ca$^{2+}$ and Na$^{+}$ or Ca$^{2+}$ and Ba$^{2+}$ showed anomalous mole fraction behaviour. We have described the current–concentration plot for Ca$^{2+}$ and Na$^{+}$ by a kinetic permeation model, i.e. the ‘step’ model.

4. Extracellular Mg$^{2+}$ blocked both divalent and monovalent currents with an IC$_{50}$ of $62 \pm 9$ $\mu$M ($n = 4$) in Ca$^{2+}$-free conditions and $328 \pm 50$ $\mu$M ($n = 4-9$) in 100 $\mu$M Ca$^{2+}$ solutions.

5. Mono- and divalent currents through ECaCs were blocked by gadolinium, lanthanum and cadmium, with a blocking order of Cd$^{2+} \gg$ Gd$^{3+} >$ La$^{3+}$.

6. We conclude that the permeation of monovalent and divalent cations through ECaCs shows similarities with L-type voltage-gated Ca$^{2+}$ channels, the main differences being a higher Ca$^{2+}$ affinity and a significantly higher current density in micromolar Ca$^{2+}$ concentrations in the case of ECaCs.

The epithelial Ca$^{2+}$ channel (ECaC) was originally cloned from rabbit kidney and is primarily expressed in the apical membrane of Ca$^{2+}$-transporting epithelia including kidney and intestine (Hoenderop et al. 1999). Together with initial electrophysiological data it has been unequivocally demonstrated that the ECaC exhibits the defining properties of a Ca$^{2+}$-selective channel which may constitute the rate-limiting step in transepithelial Ca$^{2+}$ transport (Hoenderop et al. 1999; Vennekens et al. 2000). In this sense ECaC could be the prime target for hormonal control of active Ca$^{2+}$ flux from the intestinal lumen or urine space to the blood compartment (Hoenderop et al. 2000).

The ECaC represents a new member of a large family of Ca$^{2+}$ permeable cation channels sharing homology with the transient receptor potential channel (TRPC) (Hoenderop et al. 1999). On the basis of sequence homology this group has been subdivided in three groups, i.e. STRPCs, LTRPCs and OTRPCs. The ECaC represents a new member of the latter group (Harteneck et al. 2000). This group also includes vanilloid receptor 1 (VR1) and vanilloid receptor-like 1 (VRL1), but their homology with the ECaC is low (30%), indicating that the ECaC may form another subgroup within this family of proteins. All these channels consist of six transmembrane segments including a short hydrophobic stretch between transmembrane segments 5 and 6, predicted to be the pore-forming region. This channel structure shares similarities with the core structure of the pore-forming subunits of voltage-gated Ca$^{2+}$, Na$^{+}$ and K$^{+}$ channels and with those of cyclic nucleotide-gated (CNG) channels, hyperpolarization-activated cyclic-nucleotide-gated (HCN) channels and the polycentins (PKDs) (Harteneck et al. 2000).

Electrophysiological analysis of ECaC-expressing human embryonic kidney (HEK) 293 cells demonstrated large inwardly rectifying currents which were strongly dependent on extracellular Ca$^{2+}$ and reversed at highly positive membrane potentials (Vennekens et al. 2000). The current rapidly decays during long-term Ca$^{2+}$ permeation, an effect that was significantly delayed if Ca$^{2+}$ was replaced by Ba$^{2+}$.
as charge carrier and completely abolished by lowering extracellular Ca\(^{2+}\) to 50 nm, indicating that a Ca\(^{2+}\)-dependent process inhibits ECaC activity. We have further shown that ECaCs become highly permeable to monovalent cations in the absence of extracellular Ca\(^{2+}\) (Vennekens et al. 2000). These findings point to some similarities between ECaCs and voltage-gated Ca\(^{2+}\) channels (VGCCs), which might be reflected in analogous permeation mechanisms. The aim of the present study was, therefore, to further investigate the cationic permeation mechanism of ECaC and its block by divalent or trivalent cations, and to describe the obtained data with a permeation model previously developed for voltage-gated Ca\(^{2+}\) channels.

**METHODS**

Vector construction for ECaC–GFP co-expression

The open reading frame of rbECaC was cloned as a *Pvu*II–*BamHI* fragment in the pCINeo/IRES-GFP vector (Troout et al. 1997; Vennekens et al. 2000). This bicistronic expression vector pCINeo/IRES-GFP/rbECaC was used to co-express rbECaC and enhanced green fluorescent protein (GFP).

**Cell culture and transfection**

All experiments were performed using ECaC-expressing HEK 293 cells. The cells were grown in DMEM containing 10% (v/v) human serum, 2 mm L-glutamine, 2 U ml\(^{-1}\) penicillin and 2 mg ml\(^{-1}\) streptomycin at 37 °C in a humidity controlled incubator with 10% CO\(_2\). HEK 293 cells were transiently transfected with the pCINeo/IRES-GFP/rbECaC vector using methods described previously (Kamouchi et al. 1999; Vennekens et al. 2000). Approximately 24 h after transfection, cells were used for experiments. Transfected cells were visually identified in the patch-clamp set-up. GFP was excited at a wavelength between 450 and 490 nm and the emitted light was passed through a 520 nm long-pass filter. The ECaC-expressing cells were identified by their green fluorescence and GFP-negative cells from the same batch were used as controls.

Similar results were obtained with cells expressing only GFP and GFP-negative cells.

**Electrophysiology**

Electrophysiological methods have previously been described in detail (Vennekens et al. 2000). Whole-cell currents were measured with an EPC-9 (HEKA Elektronik, Lambrecht, Germany, sampling rate 1 ms, eight-pole Bessel filter 2.9 kHz) or an L/M-EPCL-7 (List Elektronik, Darmstadt, Germany) using ruptured

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**Figure 1. Ca\(^{2+}\) currents through ECaCs in the absence of extracellular Na\(^{+}\)**

*Figure 1. Ca\(^{2+}\) currents through ECaCs in the absence of extracellular Na\(^{+}\)*

A, the effect of applying 10 mm Ca\(^{2+}\) (horizontal bar) during a 10 s step to −30 mV from a holding potential (V\(_{h}\)) of +20 mV, in the absence of extracellular Mg\(^{2+}\) and with Na\(^{+}\) replaced by NMDG\(^{+}\). ECaC-expressing HEK 293 cells were loaded with 10 mm BAPTA via the pipette in all experiments. B, current traces recorded in different Ca\(^{2+}\) concentrations (in mm) as indicated, in the absence of extracellular Mg\(^{2+}\) and with Na\(^{+}\) replaced by NMDG\(^{+}\), in response to 60 ms voltage steps to −140 mV from a V\(_{h}\) of +20 mV. C, currents recorded in different Ca\(^{2+}\) concentrations as indicated in the absence of extracellular Mg\(^{2+}\) and with Na\(^{+}\) replaced by NMDG\(^{+}\) in response to 50 ms voltage ramps from −100 to +100 mV with a V\(_{h}\) of +20 mV. D, pooled reversal potentials from 3–5 cells derived from voltage ramps as above. Currents were corrected for the capacitance current. The continuous line represents the fit of the data points with the Nernst equation (slope, 21 ± 4; n = 3–5).
patches. Electrode resistances were between 2 and 5 MΩ, and capacitance and access resistance were monitored continuously. The ramp protocol consisted of linear voltage ramps changing from −100 or −150 to +100 mV within 400 ms applied every 5 s. The step protocol consisted of a series of 60 ms voltage steps between +60 and −140 mV with a decrement of 40 mV. Holding potential was always +20 mV. Reported current densities were calculated from the current at −80 mV during the ramp protocol.

Mono-exponential fits of currents were performed using the fitting routine of the WinASCD program (G. Droogmans, KULeuven). Dose–inhibition data were fitted to a logistic dose–response function using Origin 6.0 software (MicroCal Software, Northampton, MA, USA).

**Solutions**

The standard extracellular (Krebs) solution contained (mM): 150 NaCl, 6 CsCl, 1 MgCl₂, 1·5 CaCl₂, 10 Hepes and 10 glucose, adjusted to pH 7·4 with CsOH. Na⁺-free conditions were obtained using NMDG-Cl instead of NaCl. The concentration of Ca²⁺, Ba²⁺, Sr²⁺ or Mn²⁺ was varied between 1 and 100 mM as indicated in the text. In 'nominal divalent cation-free solutions', Ca²⁺ and Mg²⁺ were omitted from Krebs solution. In these conditions the concentration of free Ca²⁺ and Mg²⁺ was estimated to be in the order of 50 nM each by means of Fura-2 measurements. In order to remove divalent cations completely, 5 mM EDTA or EGTA were added. Various Ca²⁺- and Mg²⁺-containing solutions were prepared from this buffer as indicated in the text. The amounts of Ca²⁺ and Mg²⁺ to be added were calculated with the CaBuf program (G. Droogmans, KULeuven). The intracellular (pipette) solution in all experiments contained (mM): 20 CsCl, 100 cesium aspartate, 1 MgCl₂, 10 BAPTA, 4 Na₄ATP, 10 Hepes, adjusted to pH 7·2 with CsOH.

**RESULTS**

Ca²⁺ currents through ECaCs in the absence of Na⁺

We have previously reported that the ECaC, heterologously expressed in HEK 293 cells, is an inwardly rectifying Ca²⁺ permeable channel that shows a prominent monovalent permeability in divalent cation-free solutions (Vennekens et al. 2000). In order to demonstrate that Ca²⁺ is indeed the main current carrier in high Ca²⁺ solutions, we have measured currents through ECaCs in the absence of extracellular Na⁺. Figure 1A illustrates the effect of adding 10 mM Ca²⁺ to a divalent cation-free solution containing 150 mM NMDG⁺ instead of Na⁺, during a 10 s step from +20 to −30 mV. The current amplitude shows a sharp increase followed by a rapid and reversible reduction, as was shown before in the presence of Na⁺ (Vennekens et al. 2000). This decay process is Ca²⁺ dependent, but its mechanism is not yet understood. To minimize this decay process in Ca²⁺-containing solutions, we limited the exposure time to high [Ca²⁺]ₐ to 5 s and recorded a single current trace (step or ramp) before switching back to Ca²⁺-free solution.

Figure 1B shows current traces recorded in this way in response to voltage steps to −140 mV in 1, 10, 30 and 100 mM Ca²⁺ solutions in the absence of Mg²⁺ and with Na⁺.
replaced by NMDG⁺. It is clear from this that we have to distinguish between (a) the relatively slow Ca²⁺-dependent current decay process (in Fig. 1A) and (b) the fast Ca²⁺-dependent inactivation process (in Fig.1B) (see also Vennekens et al. 2000). The amplitude of these currents increased upon increasing \([Ca^{2+}]_o\) with a concomitant shift of their reversal potential to more positive potentials, as illustrated by current traces in response to linear voltage ramps from −100 to +100 mV (duration, 50 ms) in Fig. 1C.

Figure 1D shows pooled data of the reversal potentials of 3–5 cells as a function of the extracellular Ca²⁺ concentration.

The fit of these points to the Nernst equation (continuous line) had a slope of 21 ± 4 mV (n = 3–5 cells) per 10-fold change in \([Ca^{2+}]_o\), which is in good agreement with the theoretical value of 29 mV.

**Anomalous mole fraction behaviour**

Figure 2 shows currents in response to a voltage step protocol applied to ECaC-expressing HEK 293 cells exposed

### Table 1. Comparison of the step model for ion permeation for ECaCs and L-type Ca²⁺ channels

| Current values | 10 | 9 | 8 | -3 | 20 | 6 | -2 | -18 |
| Dang & McCleskey | 10 | 10 | -2 | -4 | 9 | 2 | -2 | -15 | 5 |

Comparison of the parameters of the step model (as multiples of \(RT\), where \(R\) is the gas constant and \(T\) the absolute temperature) applied to ECaCs and those for voltage-gated Ca²⁺ channels described in Dang & McCleskey (1998).

![Image](https://example.com/image.png)

**Figure 3. Modulation of ECaC permeation by extracellular Ca²⁺: the step model**

A, mean normalized current values measured at −80 mV during linear voltage range in various \([Ca^{2+}]_o\). Currents were normalized to the current value for the same cell in buffered divalent-free solution (1082 ± 164 pA·pF⁻¹ ranging between 295 and 3000 pA·pF⁻¹, n = between 10 and 13). The continuous line represents the current densities as predicted by a model with one high affinity binding site flanked by a low affinity binding site at each side, using the energy profiles for Ca²⁺ and Na⁺ depicted in B. The dashed and dotted lines represent the fraction of the current carried by Ca²⁺ and Na⁺, respectively. B, energy profiles of the ECaC pore along the path of the pore for Ca²⁺ and Na⁺ (as multiples of \(RT\); for details see Results). C, the predicted occupation of the ECaC pore by Ca²⁺ as a function of the Ca²⁺ concentration, i.e. the chance to find 1, 2, 3 or no Ca²⁺ ions bound within the pore (for details see Results section).
to Mg$^{2+}$-free, 150 mM Na$^+$-containing solutions buffered with EGTA at extracellular Ca$^{2+}$ concentrations ranging from 10 nM to 30 mM. It is obvious that the current amplitudes are reduced by increasing extracellular Ca$^{2+}$ from virtually no Ca$^{2+}$ to up to 100 mM, but that a further increase of extracellular Ca$^{2+}$ up to 5 mM or higher enhances it again. This finding is reminiscent of the anomalous mole fraction behaviour described for L-type voltage-gated Ca$^{2+}$ channels in cardiac (Hess et al. 1986) and frog (Almers & McCleskey, 1984) muscle and for the Ca$^{2+}$ release-activated Ca$^{2+}$ (CRAC) channel (Hoth, 1995; Lepple-Wienhues & Cahalan, 1996). This anomalous mole fraction behaviour is even more evident if we plot the current amplitude as a function of the extracellular Ca$^{2+}$ concentration (Fig. 3A, data from 10–13 cells, currents at −80 mV normalized to the corresponding current from the same cell in an EGTA-buffered Ca$^{2+}$ solution, i.e. 1082 ± 164 pA pF$^{-1}$; n = 13). It shows that 50% current inhibition occurs at a Ca$^{2+}$ concentration of about 0.2 mM, a value that is comparable to that of L-type Ca$^{2+}$ channels (0.7 mM Almers & McCleskey, 1984). In contrast, the current amplitudes at Ca$^{2+}$ concentrations above 1 mM are much larger than for voltage-gated Ca$^{2+}$ channels, which is consistent with a substantial contribution of Ca$^{2+}$ to the ECaC current in this concentration range. The continuous line in Fig. 3A represents the current as predicted by the ‘step’ model, which was previously developed for L-type Ca$^{2+}$ channels (Dang & McCleskey, 1998), using the energy profiles for Ca$^{2+}$ and Na$^+$ depicted in Fig. 3B. This step model assumes a single high affinity binding site flanked by a low affinity binding site on either side. High permeation rate is accounted for by the stepwise changes in binding affinity provided by the low affinity binding sites. The energy profile was assumed to be symmetrical with equidistant barriers and wells. The depth

Figure 4. Anomalous mole fraction behaviour for Ca$^{2+}$ and Ba$^{2+}$

A, representative whole-cell current traces recorded in response to linear voltage ramps (−100 to +100 mV; V$_{h}$, +20 mV; t, 400 ms). Various mixtures of Ba$^{2+}$ and Ca$^{2+}$ were applied regarding a total sum of 30 mM divalents extracellularly in nominal Mg$^{2+}$-free conditions, in the presence of 150 mM Na$^+$ in the bath solution. B, mean normalized current values measured at −80 mV (n = 5–10) for the different solutions applied. Current values were normalized to the value for the same cell in 30 mM Ca$^{2+}$ solution (222 ± 55 pA pF$^{-1}$; n = 10).
of the central energy well for Ca\(^{2+}\) is \(-18\,RT\), corresponding to a dissociation constant of 150 nO. The heights of the outer energy barriers, i.e. 9 and 10\,RT for Ca\(^{2+}\) and Na\(^{+}\), respectively, are in the range expected for diffusional access to and departure of ions from the pore. Other parameter values are summarized in Table 1.

The dashed and dotted lines in Fig. 3A represent the fractions of the current carried by Ca\(^{2+}\) and Na\(^{+}\), respectively. Obviously, the current is mainly carried by Ca\(^{2+}\) at concentrations exceeding 1 \,mO. The predicted occupation of the channel by Ca\(^{2+}\) as a function of the Ca\(^{2+}\) concentration is shown in Fig. 3C.

The anomalous mole fraction behaviour was less pronounced for mixtures of Ca\(^{2+}\) and Ba\(^{2+}\) in nominal Mg\(^{2+}\)-free conditions (Fig. 4). The pattern is essentially the same as reported for L-type Ca\(^{2+}\) channels, but a striking difference is the fact that the current carried by Ba\(^{2+}\) through ECaCs is much smaller than that carried by Ca\(^{2+}\) (Almers & McCleskey, 1984; Hess et al. 1986). Because of the limited number of data points, however, it was not possible to fit these data reliably to a kinetic model.

**Block of mono- and divalent cation currents by divalent and trivalent cations**

We have investigated the concentration dependence of the block by extracellular Mg\(^{2+}\) on currents carried by either monovalent cations in Ca\(^{2+}\)-free solutions or Ca\(^{2+}\) in 100 \,\muM Ca\(^{2+}\)-containing solutions (Fig. 5). Figure 5A shows the time course of a typical experiment at −80 mV during sequential application of various extracellular Mg\(^{2+}\) concentrations in the absence of extracellular Ca\(^{2+}\). Corresponding I–V curves obtained from voltage ramp protocols at various Mg\(^{2+}\) concentrations are shown in Fig. 5B in the absence of Ca\(^{2+}\) and in Fig. 5C in the presence of 100 \,\muM Ca\(^{2+}\). The current amplitudes were measured at −80 mV to avoid the time-dependent component which is apparent in the presence of extracellular Ca\(^{2+}\) at negative potentials (Nilius et al. 2000). Current amplitudes at each Mg\(^{2+}\) concentration were

![Figure 5](image_url)

**Figure 5. Block of monovalent and Ca\(^{2+}\) currents through ECaCs by extracellular Mg\(^{2+}\)**

A, time course of the current at −80 mV in divalent cation-free bath solution containing 5 mM EDTA. Different Mg\(^{2+}\) concentrations were applied as indicated by horizontal bars (expressed in mM). Linear voltage ramps as in B were applied every 5 s. B, representative current traces at different extracellular Mg\(^{2+}\) concentrations, as indicated, in the absence of extracellular Ca\(^{2+}\). Cells were loaded with 10 mM RAPTA through the patch pipette. Linear voltage ramps from −100 to +100 mV were applied every 5 s (\(V_{h}\) +20 mV, \(t\), 400 ms). C, representative current traces for the Ca\(^{2+}\) current at different extracellular Mg\(^{2+}\) concentrations, as indicated, in the presence of 100 \,\muM extracellular Ca\(^{2+}\). Cells were loaded with 10 mM RAPTA through the patch pipette. Ramp protocol as in B. D, dose–response curve of Mg\(^{2+}\) blocking monovalent and 100 \,\muM Ca\(^{2+}\) currents. Mean current density at −80 mV was 1085 ± 73 pA \,\muF\(^{-1}\) (\(n\) = 4) in buffered divalent cation-free solution (○) and 328 ± 68 pA \,\muF\(^{-1}\) (\(n\) = 11) in 100 \,\muM Ca\(^{2+}\), 0 Mg\(^{2+}\) solution (▲). IC\(_{50}\) for inhibition of the monovalent current is 62 ± 9 \,\muM (\(n\) = 4), in comparison with 328 ± 50 \,\muM (\(n\) between 9 and 4) for the Ca\(^{2+}\) current. Currents were normalized to the corresponding value for the same cell in Mg\(^{2+}\)-free solution.
normalized to the corresponding current amplitude in a Mg\(^{2+}\)-free solution from the same cell (mean current density amounted to 1085 ± 73 pA pF\(^{-1}\) with \(n = 4\) for divalent-free solutions buffered with 5 mM EDTA and 328 ± 68 pA pF\(^{-1}\) (\(n = 11\)) in the presence of 100 \(\mu M\) Cd\(^{2+}\)). The pooled data, as summarized in Fig. 5D, were fitted to a logistic dose–response function. The IC\(_{50}\) values for the Mg\(^{2+}\) block (at −80 mV) were 62 ± 9 \(\mu M\) (\(n = 4\)) for the monovalent cation current and 328 ± 50 \(\mu M\) (\(n = 4–12\)) for the current mainly carried by Ca\(^{2+}\) ions. Cadmium is another divalent inorganic compound that is frequently used as a blocker of Ca\(^{2+}\) channels. It also blocked divalent currents through ECaCs (in the presence of 100 \(\mu M\) Ca\(^{2+}\)) with an IC\(_{50}\) of 2·5 ± 0·6 \(\mu M\) (\(n = 6\)). Monovalent currents on the other hand were more sensitive to Cd\(^{2+}\) and were blocked with an IC\(_{50}\) of about 2 nm (data not shown).

The trivalent cations Gd\(^{3+}\) and La\(^{3+}\) also inhibited ECaC currents. With 30 mM Ba\(^{2+}\) as the charge carrier IC\(_{50}\) values were (at −80 mV) 1·1 ± 0·2 \(\mu M\) (\(n = 13\)) and 4·6 ± 0·4 \(\mu M\) (\(n = 11\)), respectively (Fig. 6). Dose–response curves as shown in Fig. 6C were obtained analogously as described above for Mg\(^{2+}\). At a concentration of 1 \(\mu M\) both trivalent cations completely inhibited the current carried by monovalent cations in the absence of extracellular Ca\(^{2+}\) (not shown).

![Figure 6](image_url)
DISCUSSION

The epithelial Ca$^{2+}$ channel (ECaC), is a Ca$^{2+}$-selective channel, with a conductance sequence of Ca$^{2+}$ > Sr$^{2+}$ ≈ Ba$^{2+}$ > Mn$^{2+}$. The channel is constitutively opened and is subject to various Ca$^{2+}$-dependent regulation mechanisms still to be elucidated (Vennekens et al. 2000). The Ca$^{2+}$ permeability of the ECaC is confirmed in this work by the observation that the dependence of the reversal potential of the currents carried by Ca$^{2+}$ in the absence of monovalent cations has a slope of 21 mV per 10-fold change in Ca$^{2+}$ concentration, which is in fairly good agreement with the theoretical value predicted by the Nernst equation (i.e. 29 mV per 10-fold change in Ca$^{2+}$ concentration). Despite the presence of 10 mM BAPTA in the pipette solution a Ca$^{2+}$-activated Cl$^{-}$ current was activated upon addition of extracellular Ca$^{2+}$ in about 10% of the ECaC-expressing HEK cells. Evidently these cells were omitted from the analysis.

Permeation model for the ECaC

Permeation through ECaCs shows similarities to permeation through other Ca$^{2+}$-selective channels, such as voltage-gated Ca$^{2+}$ channels (Almers & McCleskey, 1984; Hess et al. 1986) and CRACs (Lepple-Wienhues & Cahalan, 1996). As shown previously, ECaCs become permeable to monovalent cations upon removal of extracellular Ca$^{2+}$ and Mg$^{2+}$ (Nilius et al. 2000). In the present paper we show that ECaC displays anomalous mole fraction (AMF) behaviour when mixtures of Na$^{+}$ and Ca$^{2+}$ or Ba$^{2+}$ and Ca$^{2+}$ are applied. The fact that the AMF effect is not so pronounced in the case of Ca$^{2+}$ and Ba$^{2+}$ is, according to us, due to the fact that the difference in permeability through the channel for Ca$^{2+}$ and Ba$^{2+}$ is not so great as that for monovalents and Ca$^{2+}$.

Anomalous mole fraction behaviour is generally accepted as evidence for a channel pore containing multiple binding sites occupied by permeant ions moving in single file through the channel (Hille, 1992). Two distinct kinetic models have been developed to account for this anomalous mole fraction behaviour and high ion transfer rate of the Ca$^{2+}$ channel pore in the case of L-type voltage-gated Ca$^{2+}$ channels, i.e. the 'repetition model' proposed by Almers & McCleskey (1984) and Hess et al. (1986) and the 'step model' by Dang & McCleskey (1998). The value of these models is that one can deduce general principles of ion permeation in the absence of an exact knowledge of the underlying molecular structure. In this work we have applied both models to our data but only for the step model were we able to deduce a set of parameters which provided a fairly good description of the data. The step model envisions a channel pore in which two low affinity binding sites flank a central high affinity binding site. In the absence of extracellular Ca$^{2+}$ the channel pore is available for monovalent permeation through the channel. However, in the presence of [Ca$^{2+}$]$_o$ the binding sites within the pore will preferentially bind Ca$^{2+}$, as a result of the higher binding affinity of the binding sites for Ca$^{2+}$ compared with Na$^{+}$. From the Ca$^{2+}$ occupancy plot it is clear that block of monovalent currents in nanomolar [Ca$^{2+}$]$_o$ occurs through binding of a single Ca$^{2+}$ ion in the pore when [Ca$^{2+}$]$_o$ rises to the micromolar range. The Ca$^{2+}$ flux at higher [Ca$^{2+}$]$_o$ is generated in parallel with multiple occupancy of the channel pore, although the chance of finding the ECaC pore in the triple Ca$^{2+}$ occupied state is very low. Put in a more direct way, Ca$^{2+}$ flux parallels the occupancy of the internal low affinity binding site. The drive for ion permeation results from the steps in binding affinity provided by the low affinity sites, as if the flanking sites provide stair steps for the ion to mount out of the channel pore (Dang & McCleskey, 1998). The most striking differences between the parameters for L-type Ca$^{2+}$ channels and ECaCs are the height of the inner barriers for Na$^{+}$ and the slightly higher Ca$^{2+}$ affinity of the central well in the case of ECaCs (Table 1). These features can account for the significantly higher current densities we measured in micromolar Ca$^{2+}$ concentrations in the case of ECaCs, compared with L-type Ca$^{2+}$ channels. The reasonably adequate description of our data by this model does not, however, guarantee the uniqueness of the derived parameters. Nevertheless our analysis clearly underscores the similarities in permeation properties of ECaCs and L-type voltage-gated Ca$^{2+}$ channels, properties which can be described by mechanisms which reconcile channel specificity and high Ca$^{2+}$ fluxes in a multiple occupied, single-file pore through steps in binding energy. As for the structural meaning of the high and low affinity binding sites further molecular characterization of the pore region of ECaCs is required.

Block of ECaCs by extracellular di- and trivalent cations

Previously we have shown that Mg$^{2+}$ blocks monovalent currents through ECaCs in a voltage-dependent manner. It was deduced through a Woodhull analysis that the Mg$^{2+}$ binding site is located within the channel pore, i.e. 31% within the membrane electrical field (Nilius et al. 2000). In the current work we found a 5-fold difference between the IC$_{50}$ of Mg$^{2+}$ block of monovalent (62 μM) and Ca$^{2+}$ currents (328 μM). Furthermore in the presence of 2 mM [Ca$^{2+}$], the IC$_{50}$ will shift even further to values up to 9 mM (data not shown). Such a difference in blocking capacity depending on the permeating cation seems to be a general property of the inorganic Ca$^{2+}$ channel blockers we have used in the current work (see also Ellinor et al. 1995; Carbone et al. 1997). In the case of Ca$^{2+}$ and Mg$^{2+}$ this difference cannot be explained by simple competition between both cations for binding to a common site inside the ECaC pore. The apparent binding constant of Mg$^{2+}$ in the presence of Ca$^{2+}$, given by:

$$K_{d,Mg}[Ca^{2+}] = K_{d,Mg}(0 Ca^{2+}) \times (1 + [Ca^{2+}]/K_{d,CA}),$$

where $K_{d,Mg}$ and $K_{d,CA}$ are the dissociation constants for Mg$^{2+}$ and Ca$^{2+}$, respectively, would indeed decrease 10$^3$-fold for a $K_{d,CA}$ of 0.2 μM by increasing [Ca$^{2+}$] from 0 to 100 μM, rather than 5-fold as observed in our experiments. An alternative though speculative mechanism could be that
binding of Ca\(^{2+}\) inside the pore influences the affinity of Mg\(^{2+}\) binding to its binding site. It is clear, however, that molecular identification of the Mg\(^{2+}\) binding site is necessary to further pursue this issue. On the other hand in the case of Cd\(^{2+}\) we indeed found a 10\(^2\) -fold difference between the IC\(_{50}\) value in the presence of 100 \(\mu\)M Ca\(^{2+}\) and in the absence of extracellular divalents. This finding therefore suggests that Ca\(^{2+}\) and Cd\(^{2+}\) compete for the same binding site within the ECaC pore. As a comparison, Cd\(^{2+}\) block of Ba\(^{2+}\) currents through voltage-gated Ca\(^{2+}\) channels (IC\(_{50}\), 300 nM in 40 mM Ba\(^{2+}\)) is 300 times less potent than the block of Li\(^{+}\) currents (IC\(_{50}\), 1 nM) (Ellinor et al. 1995), which further underlines the differences in pore properties between ECaCs and VGCCs.

Currents through ECaCs are also blocked by La\(^{3+}\) and Gd\(^{3+}\), well-known potent blockers of Ca\(^{2+}\) channels (Hille, 1992). The blocking sensitivity for ECaCs is comparable to that described for CRACs (Hoth et al. 1993), whereas L- and T-type Ca\(^{2+}\) channels are more sensitive (Ca\(^{2+}\) currents through T-type calcium channels: IC\(_{50}\) values of 267 nM and 1-02 \(\mu\)M for Gd\(^{3+}\) and La\(^{3+}\), respectively, Mi\(n\)ar & Eneyart, 1993). In contrast, analogues of the transient receptor potential channel, such as hTRP3 (Kamouchi et al. 1999) and dTRPL (Kunze et al. 1997), are less sensitive to La\(^{3+}\) and Gd\(^{3+}\).

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