The Single Pore Residue Asp\textsuperscript{542} Determines Ca\textsuperscript{2+} Permeation and Mg\textsuperscript{2+} Block of the Epithelial Ca\textsuperscript{2+} Channel* 

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The epithelial Ca\textsuperscript{2+} channel (ECaC), which was recently cloned from rabbit kidney, exhibits distinctive properties that support a facilitating role in transcellular Ca\textsuperscript{2+} (re)absorption. ECaC is structurally related to the family of six transmembrane-spanning ion channels with a pore-forming region between S5 and S6. Using point mutants of the conserved negatively charged amino acids present in the putative pore, we have identified a single aspartate residue that determines Ca\textsuperscript{2+} permeation of ECaC and modulation by extracellular Mg\textsuperscript{2+}. Mutation of the aspartate residue, D542A, abolishes Ca\textsuperscript{2+} permeation and Ca\textsuperscript{2+}-dependent current decay as well as block by extracellular Mg\textsuperscript{2+}, whereas monovalent cations still permeate the mutant channel. Variation of the side chain length in mutations D542N, D542E, and D542M attenuated Ca\textsuperscript{2+} permeability and Ca\textsuperscript{2+}-dependent current decay. Block of monovalent currents through ECaC by Mg\textsuperscript{2+} was decreased. Exchanging the aspartate residue for a positively charged amino acid, D542K, resulted in a nonfunctional channel. Mutations of two neighboring negatively charged residues, i.e. Glu\textsuperscript{535} and Asp\textsuperscript{550}, had only minor effects on Ca\textsuperscript{2+} permeation properties.

Transcellular Ca\textsuperscript{2+} transport in polarized epithelia of the kidney, intestine, and placenta is of vital importance for overall Ca\textsuperscript{2+} homeostasis. Recently, the rate-limiting step in this Ca\textsuperscript{2+} transport, the epithelial Ca\textsuperscript{2+} channel (ECaC), was identified and implicated as the prime candidate target for hormonal control of transcellular Ca\textsuperscript{2+} transport in these epithelia (1–6).

We have recently analyzed ECaC in extensive electrophysiological studies using Xenopus oocytes and HEK293 cells heterologously expressing ECaC, which is important to understand the Ca\textsuperscript{2+} influx in Ca\textsuperscript{2+}-transporting epithelia (5–7). The functional hallmarks of ECaC comprise a constitutively activated Ca\textsuperscript{2+}-selective cation channel with a substantial permeability at physiological membrane potentials and a Ca\textsuperscript{2+}-dependent feedback regulation of channel activity including fast inactivation and slow current decay. Interestingly, ECaC becomes permeable to monovalent cations by lowering extracellular Ca\textsuperscript{2+} to micromolar concentrations. Under these latter conditions, the single channel conductance of ECaC is ~70 picoisemiers.

ECaC is most closely, albeit still distantly, related to capsaicin receptors and the trp channel family (8–12). These channels function as Ca\textsuperscript{2+}-permeable cation channels and contain six putative transmembrane domains, including a pore-forming region between S5 and S6. Despite their structural similarity, these channels differ from ECaC in their mechanism of activation and some of their functional properties (8–11). The capsaicin receptors facilitate Ca\textsuperscript{2+} influx after receptor activation, whereas the trp channels are activated after Ca\textsuperscript{2+} store depletion or phospholipase C activation. ECaC has a high selectivity for Ca\textsuperscript{2+}, illustrated by P\textsubscript{Ca/PNa} values of more than 100, whereas the homologous channels display in general a less eminent Ca\textsuperscript{2+} selectivity. Furthermore, the current-voltage relationship of the capsaicin receptor and trp channels displays outward rectification, whereas ECaC currents show a pronounced inward rectification.

The aim of the present study was to elucidate the molecular determinants responsible for the key properties of ECaC. Only three negatively charged amino acid residues reside in the putative pore-forming region of ECaC, i.e. Glu\textsuperscript{535}, Asp\textsuperscript{542}, and Asp\textsuperscript{550}, which are potential Ca\textsuperscript{2+}-binding sites determining the conductive properties of this channel. Interestingly, these residues are absent in the other structurally related six transmembrane-spanning proteins, except for Glu\textsuperscript{535}, which is conserved in the capsaicin receptor (2, 4) and could therefore be responsible for the distinguishable features of ECaC. We have evaluated the functional role of these negatively charged residues and show that the aspartate at position 542 is crucial for the described hallmarks of ECaC. These findings are of vital importance in our understanding of how ECaC can show an exquisite Ca\textsuperscript{2+} selectivity that is relevant for the Ca\textsuperscript{2+} handling by Ca\textsuperscript{2+}-absorbing epithelia and might be important to understand dysfunctioning of Ca\textsuperscript{2+} absorption.

EXPERIMENTAL PROCEDURES

Molecular Biology—The open reading frame from rbECaC was cloned as a P\textsubscript{nuI}-BamHI fragment in the pCIneo/IRESGFP vector (2, 4, 13). We used this bicistronic expression vector, pCIneo/IRESGFP/ rbECaC, to coexpress rbECaC and enhanced GFP. Mutagenesis of the amino acids at positions 535, 542, and 550 was carried out using the QuickChange\textsuperscript{®} Site-directed Mutagenesis Kit (Stratagene). The nucleotide sequences of mutants D\textsubscript{535}A, D\textsubscript{542}A, D\textsubscript{542}E, D\textsubscript{542}N, D\textsubscript{542}K, and D\textsubscript{550}A have been verified by sequencing the corresponding cDNAs.

Cell Culture and Transfection—Human embryonic kidney cells
for a maximum of 5 min to a Krebs solution containing 1.5 mM Ca2+

tions of mannitol to the Krebs or Ca2+

uously. The internal (pipette) solution contained 20 mM CsCl, 100 mM

patches. Cell capacitance and access resistance were monitored contin-

ned with an EPC-9 (HEKA Elektronik, Lambrecht, Germany) or an

ance was between 2 and 5 megaohms. Whole-cell currents were meas-

ments have been described in detail previously (15). Electrode resist-

formed at room temperature (20 °C–22 °C). Stimulation protocols con-

, N,N,N9,N9-tetraacetic acid, 4 mM Na2ATP, and 10 mM HEPES, pH 7.2,

tions are in gray boxes

were grown in Dulbecco’s modified Eagle’s medium containing 10% (v/v) human serum, 2 mM l-glutamine, 2 units/ml penicillin,

and 2 mg/ml streptomycin at 37 °C in a humidity-controlled incubator

Fig. 1. Alignment of ECaC pore region with that of homologous channels. Identical residues are in black boxes, conservative substitu-

Fig. 2 shows the effects of mutating aspartate at position 542 for alanine on the kinetics of ECaC currents during hyperpolarizings

Functional Characterization of the Single Point Mutant

RESULTS

Pore Mutations in ECaC

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Fig. 2. Phenotypes of the currents through wild-type ECaC and the D542A mutant channel. Currents during voltage steps ranged from +60 mV to −140 mV (decrement, −40 mV; holding potential, +20 mV) in nominally divalent cation-free solution and solution plus either 1 mM Mg²⁺, 1.5 mM Ca²⁺ and 1 mM Mg²⁺, or 0.1 mM EDTA. Data were obtained from HEK cells transfected with wild-type ECaC (A) or D542A (B). C and D, currents in response to a voltage ramp protocol from −150 to +100 mV (V_holding, +20 mV; duration, 400 ms). In the absence of extracellular monovalent cations (thin line, all substituted by N-methyl-D-glucamine⁻), administration of 30 mM Ca²⁺ (thick line) results in a large inward current carried by Ca²⁺ in the absence of another permeable cation. In the D542A mutant, the background current in monovalent cation-free solution was even inhibited by administration of 30 mM Ca²⁺.

panel from the right). Cells expressing the D542A mutant displayed large currents, which showed intrinsic slow inactivation. In contrast to wild-type ECaC, these currents were completely independent of extracellular Mg²⁺ and Ca²⁺ (Fig. 2B). The rapid current decay component due to Mg²⁺ block was absent in D542A, indicating that this mutant is insensitive to block by Mg²⁺. This was further supported by the lack of an EDTA effect (Fig. 2B, right panel). A more detailed analysis revealed that the permeation pattern, estimated from the currents at −80 mV normalized by the size Na⁺ currents at this potential, of monovalent cations through D542A was also different from that of wild-type ECaC. The permeation sequence of wild-type ECaC was Eisenman X, with Na⁺ being more permeable than Li⁺ (Fig. 3A). D542A mutants, however, were more permeable for Li⁺ than for Na⁺, which is consistent with an Eisenman XI sequence (Fig. 3, B and C). The Ca²⁺ permeability of ECaC can be directly shown when Ca²⁺ is added to an extracellular solution in which all monovalent cations were substituted by N-methyl-D-glucamine⁻. Under these conditions, only small currents remained, which were likely Cl⁻ currents. Administration of Ca²⁺ induced a large, inwardly rectifying current that reversed at very positive potentials. This current indicates Ca²⁺ influx through ECaC (16). It was completely absent in D542A. On the contrary, the background currents were even blocked in the presence of Ca²⁺ (Fig. 2, C and D). Currents through wild-type ECaC showed clear inward rectification (Fig. 4A), with a density of 534 ± 137 pA/PF for 30 mM Ca²⁺ at −80 mV (n = 32). The reversal potential was shifted from +15 ± 2 mV in divalent cation-free solutions (n = 21) to +47 ± 3 mV (30 mM Ca²⁺; n = 24). For evaluation of the permeability of Ca²⁺, we measured the difference of the reversal potential in Ca²⁺-free solutions and in 30 mM Ca²⁺-containing solution for all mutants tested. The absence or negative shift of the reversal potential indicates the absence of Ca²⁺ permeability. The averaged data are summarized in Fig. 4G. The D542A mutant did not show rectification, and current densities were smaller than those in wild-type ECaC (Fig. 4, B and E) but significantly higher than those in nontransfected HEK cells (2.5 ± 0.4 pA/PF at 30 mM Ca²⁺ and −80 mV; n = 8), indicating that the mutant is functionally expressed. Extracellular divalent cations slightly shifted the reversal potential to more negative potentials, indicating that the D542 channels are no longer permeable for divalent cations (Fig. 4G). The rapid and Ca²⁺-dependent current decay during repetitive stimulations with voltage ramps, another characteristic feature of wild-type ECaC (5, 6), was abolished in the D542A mutant (Fig. 5, A and B).

Functional Effects of Mutations on Residues Neighboring Asp⁵⁵⁴—Expression of the E535A and D550A mutants induced large inwardly rectifying currents (in 30 mM Ca²⁺ and at −80 mV: 151 ± 37 pA/PF (n = 8) and 87 ± 23 pA/PF (n = 7), respectively) that are not significantly different from wild-type ECaC (Fig. 4, C and D). The Ca²⁺ permeability of both mutants...
voltage ramps from 2 Tant channels. A presence of 30 mM Ca2+ mutants express functional cation channels. The current den-

spective mutants. Results are shown in Tables I and II. All

permeation profile for monovalent cations through these re-

for VR1 could be obtained. In addition, we have studied the

rate of decay is faster for the E535A mutant and slower for the

decay comparable to that of wild-type currents, although the

was not significantly different from that of wild-type ECaC.

Fig. 5 shows that currents through both mutants show a fast
decay comparable to that of wild-type currents, although the
rate of decay is faster for the E535A mutant and slower for the
D542A mutant (Fig. 5C). As in the wild type, Mg2+ reduced the
current at the end of the hyperpolarizing pulses for both the
E535A and D550A mutant, with the block being more pro-
nounced in E535A than in D550A (Fig. 6, A, C, and D).

Remodeling the Asp542 Residue—As shown above, the aspar-
tate residue close to the pore is important for ECaC. A similar
situation was recently described for the vanilloid receptor, in
which neutralization of Asp646 reduced the permeability for
Mg2+ (12). To further elucidate the role of this crucial site, we
have mutated the aspartate residue by either glutamate, a
negatively charged amino acid with a longer side chain, the
polar asparagine, methionine with a longer side chain, and the
positively charged lysine. As shown in Fig. 1, VR1, a nonselective
channel that is also permeable for Ca2+, has a methionine
residue at the same site. Therefore, we tested this special
mutant to probe whether pore characteristics similar to those
for VR1 could be obtained. In addition, we have studied the
permeation profile for monovalent cations through these re-
spective mutants. Results are shown in Tables I and II. All
mutations express functional cation channels. The current den-

sivity is significantly increased for all mutants except D542K; the
current density for D542K did not differ from that seen in
nontransfected HEK cells (see Table I). D542K also showed the
same permeation phenotype (data not shown) as nontrans-
fected cells, indicating that no functional channels were ex-
pressed. However, the permeation profiles for monovalent
cations were clearly different in the other mutants. In
nontransfected cells, the permeation sequence follows Eisen-
man IV for a weak field strength site (Cs > K > Na > Li).
D542M expression resulted only in very small currents with an
Eisenman type IV permeation profile, also indicating a weak
field strength binding site for cations that is equivalent to the
background cation conductance. D542E expression also re-
sulted in small currents, but the permeation type still matched
that of a high field strength binding site as observed for wild-
type ECaC, E535A, and D550A, but not in D542A. This decay was fitted
by a monoexponential function. B, time course of current decay recon-
structed from the normalized successive current amplitudes at −80 mV
fitted to a monoexponential function. C, synopsis of the time constants
of current decay in 1 and 30 mM extracellular Ca2+ concentration for the
wild type (WT), E535A, and D550A.

![Fig. 4. Permeation of Ca2+ through wild-type ECaC and mutants channels. A, current-voltage relationships obtained from linear voltage ramps from −150 mV to +100 mV for wild-type ECaC in the presence of 30 mM Ca2+ (dotted line) and in divalent-free solution. Note the positive shift in reversal potential in the presence of divalent cations as well as the inward rectification. The reversal potential in the presence of 30 mM Ca2+ is always indicated by an arrow, and the reversal potential in the presence of divalent-free solution is indicated by a solid circle. B, current-voltage relationships of the mutant D542A under the same conditions as shown in A. Note that no obvious shift in the reversal potentials occurred in the presence of divalent cations. C—F, current-voltage relationships of the E535A, D550A, D542N, and D542E mutants, respectively, using the same protocol described in A. G, shift of the reversal potentials in the presence of 30 mM Ca2+ (E revoke) from that in the absence of divalent cations (E revoke), which is used as a measure of divalent permeability through ECaC (data from 6–12 cells).](https://example.com/fig4)

![Fig. 5. Ca2+-dependent current decay in wild-type ECaC and its mutants. A, current decay during repetitive voltage ramps in the presence of 30 mM Ca2+ (ramps range from −100 mV to +100 mV; holding potential, +20 mV; interval between the ramps, 5 s) in wild-type ECaC, E535A, and D550A, but not in D542A. This decay was fitted by a monoexponential function. B, time course of current decay reconstructed from the normalized successive current amplitudes at −80 mV fitted to a monoexponential function. C, synopsis of the time constants of current decay in 1 and 30 mM extracellular Ca2+ concentration for the wild type (WT), E535A, and D550A.](https://example.com/fig5)
Pore Mutations in ECaC

The present study demonstrates that a single aspartate residue located in the putative pore region of ECaC is crucial for its key features including a high Ca\(^{2+}\) permeability, block by Mg\(^{2+}\), and Ca\(^{2+}\)-dependent current decay. These properties are essential for ECaC functioning in Ca\(^{2+}\) (re)absorption in the kidney, intestine, and placenta.

The putative pore region of ECaC contains three negatively charged amino acids that are conserved among different species (17). Our data clearly show that Asp\(^{542}\) is an important molecular determinant of Ca\(^{2+}\) selectivity and Ca\(^{2+}\) permeation through ECaC. The effects of an extension of the side chain length of this negative residue (D542E) indicate that not only the negative charge but also a steric factor controlled by the length of that side chain is important. Unexpectedly, the currents through the D542E mutants are much smaller than those for the Ala and Asn mutant. They are similar in size for the D542M mutants. D542M showed very small currents. These data, together with the permeation sequence similar to the background cation conductance, may indicate that D542M might be even nonfunctional. It is likely that the longer side chain in the D542M mutants may hinder permeation of Ca\(^{2+}\) and decrease the field strength of the monovalent binding site (Eisenman IV). Exchange of aspartate for an uncharged but polar amino acid (D542N) had similar effects, indicating that the electronegative oxygen of this side chain (at physiological pH) cannot substitute for the negative charge of aspartate. These findings indicate that both the charge and the length of the side chain at position 542 are essential for Ca\(^{2+}\) permeability.

Under physiological conditions, ECaC transports Ca\(^{2+}\) efficiently with a high degree of selectivity over monovalent cations (5). Permeation of monovalent cations follows the Eisenman X sequence, indicating a strong field strength binding site (6). Mutation of Asp\(^{542}\) into an alanine induced a shift in the permeability for monovalent cations from Eisenman X to Eisenman XI, indicating that, in contrast with the permeation of Ca\(^{2+}\), the binding of monovalent cations is not influenced, or that the field strength of the binding site is even increased.

There is no evidence for additional negatively charged residues in the pore region. The existence of a second putative binding site for Ca\(^{2+}\) in another region of the pore is therefore unlikely. It is therefore difficult to reconcile the observed anomalous mole fraction behavior of ECaC (5) with the two-binding site model used to describe Ca\(^{2+}\) selectivity and high Ca\(^{2+}\) permeability of voltage-operated Ca\(^{2+}\) channels.

It is remarkable that substitution of aspartate at position

![Diagram]

**Fig. 6.** Mg\(^{2+}\) block of monovalent currents through wild-type ECaC and its mutants. A-F, current responses to a hyperpolarizing voltage pulse from +20 to −140 mV in divalent cation-free solutions and in the presence of 1 mM Mg\(^{2+}\) in wild-type ECaC (WT), D542A, E535A, D550A, D542E, and D542N. The rapid decay compared with divalent cation-free solutions is due to voltage-dependent block of ECaC by Mg\(^{2+}\). G, Mg\(^{2+}\) block was calculated as the ratio of the current at the end of the voltage pulse in 1 mM Mg\(^{2+}\) and that in the absence of divalent cations. Block is present in wild-type ECaC and in all mutants except D542A (pooled data from 5–14 cells are shown).

**TABLE I**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Na(^{+}) current density (pA/pF)</th>
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<tbody>
<tr>
<td>Nontransfected</td>
<td>5.7 ± 2</td>
<td>17</td>
</tr>
<tr>
<td>Wild-type</td>
<td>487 ± 76</td>
<td>16</td>
</tr>
<tr>
<td>D542A</td>
<td>402 ± 110</td>
<td>14</td>
</tr>
<tr>
<td>D542N</td>
<td>317 ± 55</td>
<td>16</td>
</tr>
<tr>
<td>D542E</td>
<td>46 ± 18</td>
<td>12</td>
</tr>
<tr>
<td>D542M</td>
<td>43 ± 15</td>
<td>17</td>
</tr>
<tr>
<td>D542K</td>
<td>17 ± 5</td>
<td>6</td>
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</tbody>
</table>

**TABLE II**

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<th>Mutant</th>
<th>K(^{+})</th>
<th>Cs(^{+})</th>
<th>Li(^{+})</th>
<th>n (^{a})</th>
<th>Type</th>
<th>Eisenman sequence</th>
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<tr>
<td>NT(^{b})</td>
<td>1.91 ± 0.26</td>
<td>1.21 ± 0.05</td>
<td>0.72 ± 0.04</td>
<td>7</td>
<td>K &gt; Cs &gt; Na &gt; Li</td>
<td>IV</td>
</tr>
<tr>
<td>WT</td>
<td>0.59 ± 0.04</td>
<td>0.46 ± 0.04</td>
<td>0.82 ± 0.04</td>
<td>8</td>
<td>Na &gt; Li &gt; K &gt; Cs</td>
<td>X</td>
</tr>
<tr>
<td>D542A</td>
<td>0.82 ± 0.06</td>
<td>0.73 ± 0.05</td>
<td>1.33 ± 0.07</td>
<td>8</td>
<td>Li &gt; Na &gt; K &gt; Cs</td>
<td>XI</td>
</tr>
<tr>
<td>D542N</td>
<td>0.58 ± 0.05</td>
<td>0.36 ± 0.04</td>
<td>0.92 ± 0.07</td>
<td>5</td>
<td>Na &gt; Li &gt; K &gt; Cs</td>
<td>X</td>
</tr>
<tr>
<td>D542E</td>
<td>0.75 ± 0.03</td>
<td>0.62 ± 0.03</td>
<td>1.27 ± 0.08</td>
<td>5</td>
<td>Li &gt; Na &gt; K &gt; Cs</td>
<td>XI</td>
</tr>
<tr>
<td>D542M</td>
<td>2.78 ± 0.61</td>
<td>1.1 ± 0.07</td>
<td>0.71 ± 0.04</td>
<td>5</td>
<td>K &gt; Cs &gt; Na &gt; Li</td>
<td>IV</td>
</tr>
</tbody>
</table>

\(^{a}\) n = number of cells.

\(^{b}\) NT, nontransfected cells; WT, wild-type ECaC.

**Relative permeability of monovalent cations through ECaC**

Relative permeability for various monovalent cations trough ECaC as measured from reversal potential shifts (Eq. 1). Shifts were always measured from the same cells.
542 by glutamate attenuated the Ca\(^{2+}\) permeability of ECaC because conserved glutamate residues form the high-affinity binding site in voltage-operated Ca\(^{2+}\) channels. The pore region of the vanilloid receptor family is homologous to ECaC but lacks the aspartate at 542, which may explain the observed low Ca\(^{2+}\) selectivity of these channels (9, 10). Instead, methionine is placed at this site. The D542M mutation in ECaC only results in small currents. The pore is still permeable for monovalent cations with an Eisenman type IV permeation sequence, indicating a weaker field strength binding site than that seen for all the other mutants. However, in contrast with VR1, which is permeable for Ca\(^{2+}\) and Mg\(^{2+}\), the mutant D542M channel is impermeable for Ca\(^{2+}\), indicating that the mechanism of permeation through the ECaC pore is different from that of VR1. It is interesting that a homologous member of this family cloned from rat intestine, calcium transporter 1, contains an additional negative aspartate between the two aspartate residues at positions 542 and 550 (16). This additional aspartate could produce subtle differences in Ca\(^{2+}\) permeability of these channels with important implications for Ca\(^{2+}\) handling in the respective tissues, but electrophysiological data about calcium transporter 1 are currently lacking to substantiate this concept.

Obviously, aspartate at position 542 is also essential for Mg\(^{2+}\) binding and channel block. In addition, Mg\(^{2+}\) block of the monovalent current through ECaC still persists when the side chain of the negatively charged residue is prolonged (D542E) or by substitution of the aspartate into the polar asparagine (D542N). Thus, the structure of the Mg\(^{2+}\)-binding site may differ from that of the Ca\(^{2+}\)-binding site. The fact that E535A increases and D550A decreases the block by Mg\(^{2+}\) indicates that Mg\(^{2+}\) binding to the essential Asp\(^{542}\) site is modulated by these adjacent negative charges.

In conclusion, the molecular determinants of Ca\(^{2+}\) selectivity and permeation of ECaC appear to reside at a single aspartate residue in the pore region, a role that is played by glutamate residues in voltage-operated Ca\(^{2+}\) channels. It is therefore tempting to speculate that the selectivity filter for Ca\(^{2+}\) in ECaC might also consist of a ring of four negatively charged residues in a tetrameric pore molecule. Consequently, single mutations in the pore of ECaC would cause defective channels, which would be unable to perform a significant Ca\(^{2+}\) absorptive function. It will certainly be challenging to search for such mutants in patients with familiar malabsorption of Ca\(^{2+}\).

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REFERENCES