Cadmium Trapping in an Epithelial Sodium Channel Pore Mutant*

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The putative selectivity filter of the epithelial sodium channel (ENaC) comprises a three-residue sequence G/SXS, but it remains uncertain whether the backbone atoms of this sequence or whether their side chains are lining the pore. It has been reported that the S589C mutation in the selectivity filter of αENaC renders the channel sensitive to block by externally applied Cd2+; this was interpreted as evidence for Cd2+ coordination with the thiol group of the side chain of α589C, pointing toward the pore lumen. Because the α5589C mutation alters the monovalent to divalent cation selectivity ratio of ENaC and because internally applied Cd2+ blocks wild-type ENaC with high affinity, we hypothesized that the inhibition of α5589C ENaC by Cd2+ results rather from the coordination of this cation with native cysteine residues located in the internal pore of ENaC. We show here that Cd2+ inhibits not only ENaC α5589C and α5589D but also α5589N mutants and that Ca2+ weakly interacts with the S589D mutant. The block of α5589C, -D, and -N mutants is characterized by a slow on-rate, is nearly irreversible, is voltage-dependent, and can be prevented by amiloride. The C546S mutation in the second transmembrane helix of γ subunit in the background of the ENaC α5589C, -D, or -N mutants reduces the sensitivity to block by Cd2+ and renders the block rapidly reversible. We conclude therefore that the block by Cd2+ of the α5589C, -D, and -N mutants results from the trapping of Cd2+ ions in the internal pore of the channel and involves Cys-546 in the second transmembrane helix of the γENaC subunit.

The epithelial sodium channel (ENaC)2 mediates the Na+ ion influx across the apical membrane of tight epithelia such as the aldosterone-sensitive distal nephron, the distal colon, or the airways epithelium (1). In principal cells of the aldosterone-sensitive distal nephron, the ENaC-mediated influx of Na+ ions constitutes the apical step of the transepithelial Na+ absorption and is regulated by aldosterone (2). The aldosterone-sensitive distal nephron plays an important role in the maintenance of Na+ homeostasis, the regulation of extracellular volume, and blood pressure (3). In the lungs, ENaC together with cystic fibrosis transmembrane conductance regulator controls the height of the airway surface liquid that forms a thin layer coating the airways epithelium, which contributes to the clearance of particles and microbes (4).

Functional ENaC at the cell plasma membrane is a heteromeric protein made of homologous α, β, and γ subunits arranged pseudosymmetrically around a central channel pore, presumably in a 2α, 1β, and 1γ configuration (5, 6). Each ENaC subunit is made of two transmembrane helices (TM1 and TM2), a large extracellular loop that represents more than half of the mass of the protein, and intracellular N and C termini. The ENaC subunits belong to the ENaC/degenerin superfamily of ion channels but share no sequence homology with other tetrameric channels also made of two transmembrane domains, such as inward rectifier K+ channels.

In the absence of crystal structures of members of the ENaC/degenerin ion channel family, our understanding of mechanisms and structures involved in the ion permeation is quite limited. The amino acid sequence preceding the second transmembrane domain (TM2) constitutes the outer channel pore (pore region in Fig. 1) where the pore blocker amiloride binds (αSer-583, βGly-525, γGly-537 in rat ENaC sequence, see Fig. 1) (7). Secondary structure predictions conform the TM2 to an α helix (8). The amiloride binding site is located external and upstream to the selectivity filter with an amino-to-carboxyl sense. The conserved three-amino acid sequence G/SXS (587–589 in rat ENaC) that is essential to maintain the high selectivity of ENaC for Na+ over K+ or Ca2+ ions is considered therefore a key element of the selectivity filter (9). It has been proposed that the increase in permeability to K+ and NH4+ resulting from mutations of αSer-589 reflects changes in the geometry of the pore segment comprising the selectivity filter that allow it to accommodate larger cations (10). The molecular basis of the interaction between the permeant Na2+ or Li2+ ions and oxygen atoms at the binding site is not yet clear. Recent experiments reported that the serine 589 to cysteine substitution in the α subunit (αS589C) confers to ENaC a sensitivity to block by external Cd2+ ions, which was interpreted as evidence for an orientation of the side chain of serine 589 toward the lumen of the channel pore (11).

ENaC and the K+ channel KcsA share a similar membrane topology and a heterotetrameric structure made of homologous subunits comprising two transmembrane segments. The
The conduction pore of ENaC appears to be, however, quite different from that of the members of the K⁺ channel superfamily. The amino-terminal portion of the pore region in the KcsA forms an α helix (pore helix) pointing from the outside toward the center of the channel (12). Following this pore helix, the amino acid stretch lining the selectivity filter folds back toward the external surface, exposing binding sites for external blocking ligands such as toxis or triethanolamine (TEA). In ENaC, the pore region points from the outside to the center of the channel with an amino-to-carboxyl sense of, successively, the amiloride binding site, the selectivity filter, and the TM2 (1).

These important differences in the pore organization indicate that the three-dimensional structure of the KcsA K⁺ channel does not represent an adequate template to understand ion permeation through ENaC. The pore-lining residues beyond the selectivity filter that constitute the internal channel pore remain to be identified in channels belonging to the ENaC/degenerin family, for which only a few studies have been performed to determine the accessibility of inner pore lining residues. Recent work has shown that, in contrast to the acid-sensing ion channel (ASIC), another member of the ENaC/degenerin family, ENaC is highly sensitive to intracellularly applied sulfhydryl reagents such as Cd²⁺, Zn²⁺, or methanethiosulfonates (MTS). Cysteine residues in the N terminus of ENaC contribute to inhibition by MTS reagents (13). Similar inhibition of ASIC by internal sulfhydryl reagents can be reproduced in ASIC mutants in which cysteines have been introduced at the N terminus and in the S'-start of the TM2 (14). Altogether, these observations suggest that the N terminus and the internal part of TM1 participate in the pore lining of ENaC.

In this study we have revisited the mechanism of channel blockade by external Cd²⁺ in ENaC mutants with amino acid substitutions in the selectivity filter (αS589C) (11). We show that, in order to block ENaC, Cd²⁺ has to bind to distinct sites deep in the channel pore, including a cysteine residue in the TM2 of γENaC subunits.

**MATERIALS AND METHODS**

**Site-directed Mutagenesis, Expression in Xenopus laevis Oocytes, and Electrophysiology**—The ENaC constructs used for this study were taken from a previous study (10, 13). Complementary RNAs of each α, β, γ subunit were synthesized in vitro with SP6 RNA polymerase from wild-type and mutant α, β, and γENaC cDNA encoding vectors previously linearized with PvuII (α and γ subunits) or BglII (β subunit).

Healthy stage V and VI X. laevis oocytes were pressure-injected with 100 nl of a solution containing equal amounts of α, β, and γ ENaC subunits at a total concentration of 100 ng/µl. For standard experiments, oocytes were kept at 19°C in a low Na⁺ modified Barth’s saline: in mM, 10 NaCl, 0.82 MgSO₄, 0.41 CaCl₂, 0.33 Ca(NO₃)₂, 80 NMDG, 2 KCl, and 5 HEPES, pH 7.2. Electrophysiological measurements were made 16–30 h after injection. ENaC current (I Na) was recorded using the two-electrode voltage-clamp technique (model TEV-200; Dagan Corp.). Holding potential was −100 mV. The amiloride-sensitive currents defined as the difference in I measured before and after addition of 10 µM amiloride (Sigma-Aldrich) in the bath were considered ENaC-mediated macroscopic I Na. All electrophysiological experiments were performed at room temperature (22–25°C). Oocytes were maintained in a recording chamber continuously perfused with a standard bath solution containing in mM, 120 NaCl, 2.5 KCl, 1.8 CaCl₂, 2H₂O, and 10 HEPES-NaOH, pH 7.2. For cadmium block experiments, 0.01, 0.1, 1, or 10 mM Cd²⁺ was freshly added to the perfusion solution. Results are reported as means ± S.E. and represent the mean of n independent experiments in which the average amiloride-sensitive Na⁺ current I Na was measured for four to ten individual oocytes originating from different frogs.

**Analysis of the Data**—Data obtained from the time course of I Na decrease in the presence of external Cd²⁺ were best fitted by the sum of two exponentials. To analyze the voltage dependence curve of the I Na current of the ENaC mutant, the ratio I/I₀ measured in presence (I) of 1 or 3 mM Cd²⁺ to that in the absence of the Cd²⁺ (I₀) is described by the Woodhull equation (15) as shown in Equation 1

\[
P_\text{on} = I/I_0 = K_0 \exp(z'FV/RT)/(K_0 + K_b \exp(z'FV/RT)) \quad (\text{Eq. 1})
\]

where P_on is the probability that the binding site of Cd²⁺ is unblocked; K₀ is the equilibrium dissociation constant at 0 mV; [B] is the blocker concentration; z' is the slope parameter; F, the Faraday’s constant; V, the applied voltage; R, the gas constant, and T, the absolute temperature. Moreover, z' is equal to the product of the actual valence of the blocking ion and the fraction of the membrane potential (or electrical distance) δ acting on the ion: z' = zδ.

**RESULTS**

It has recently been reported that serine-to-cysteine substitution at position 589 in αENaC makes the channel inhibitable by external Cd²⁺ (11). The block of ENaC αS589C mutant by Cd²⁺ shows an unusual slow on-rate (k_on > 1000 M⁻¹ s⁻¹), as well as a slow and incomplete recovery of ENaC activity after external Cd²⁺ removal. This was interpreted as evidence for Cd²⁺ coordination with the thiol group of the side chain of the cysteine at position αS589 (11).

αSer-589 is located in the selectivity filter sequence G₅₈₉S₅₈₈S₅₈₉ of rat ENaC (see Fig. 2), and its mutation drastically changes the ion selectivity of the channel (9, 10). We hypothesized that these unusual characteristics of block by Cd²⁺ are due to the trapping of Cd²⁺ ions in the internal pore of the channel, i.e. downstream the selectivity filter as far as the channel opening to the cytosol. Two previous observations support this hypothesis. First, the αS589C mutation makes the...
channel permeable to divalent cations such as Ca\(^{2+}\) and possibly also to Cd\(^{2+}\), which has a smaller ionic radius than the former (9). Second, we have recently reported that, in both inside-out patch and in cut-open oocytes, Cd\(^{2+}\) applied at micromolar concentrations to the intracellular side of ENaC blocks the channel; this block was characterized by a slow and partial recovery upon removal of Cd\(^{2+}\) (13).

To determine the requirement of a thiol side chain at position S583 for the binding of external Cd\(^{2+}\) and the inhibition of ENaC, we tested its effect on different substitution mutants, e.g. αS589C, αS589D, αS589N, and αS589A. Cd\(^{2+}\) ions are known to bind the sulphydryl group of cysteine or the fully charged oxygen atoms contributed by glutamate or aspartate side chains; however, Cd\(^{2+}\) has little affinity for side chains of asparagine. Fig. 2 shows representative recordings of ENaC-mediated amiloride-sensitive currents (\(I_{\text{Na}}\)) and their inhibition by external Cd\(^{2+}\). Cadmium at 0.1 or 1 mM inhibits the \(I_{\text{Na}}\) generated by ENaC αS589C, αS589D, and αS589N mutants (Fig. 2, A–C). A higher concentration of Cd\(^{2+}\) (10 mM) is required to inhibit the αS589A mutant (Fig. 2D), whereas wild-type ENaC remains insensitive to inhibition by Cd\(^{2+}\) (Fig. 2E).

The blockade of ENaC αSer-589 mutants by Cd\(^{2+}\) slowly reaches equilibrium. This is particularly evident for low concentrations of Cd\(^{2+}\) and for the αS589N mutant, which appears slightly less sensitive to Cd\(^{2+}\) than the αS589C and αS589D counterparts. It was difficult to determine the true affinity of the αSer-589 mutants for Cd\(^{2+}\) because at low Cd\(^{2+}\) concentrations the blockade equilibrium was still not reached after several minutes. At a higher concentration of Cd\(^{2+}\) (1 mM) the rate of channel inhibition is faster and the block almost complete after 1 min. This contrasts with the fast kinetics of inhibition of the αS583C ENaC mutant by Cd\(^{2+}\) at concentrations varying from 0.01 to 1 mM (\(IC_{50} = 0.105 \pm 0.0012\) mM, \(n = 10\): external Cd\(^{2+}\) rapidly reduces the current level in a dose-dependent manner without changes in the on-rate of channel blockade (Fig. 3). By contrast to the αS589N, the αS583N mutant is insensitive to inhibition by external Cd\(^{2+}\). There is strong evidence that the side chain of the cysteine at position Ser-583 is oriented toward the channel lumen, allowing Cd\(^{2+}\) to bind in the external pore and to block the channel (16, 17). It also shows that Asn does not bind Cd\(^{2+}\) at any of the assayed concentrations, and we conclude therefore that the lower sensitivity of the αS589N mutant to Cd\(^{2+}\) inhibition probably results from a decreased permeability to this cation as compared with that of the other αS589 mutants. Moreover, the discrepancy between the αSer-589 and αSer-583 mutants regarding their sensitivity to Cd\(^{2+}\) and the kinetics of block indicate that the mechanism of ENaC inhibition by Cd\(^{2+}\) is different for the two mutants.

The on-rate of channel inhibition by Cd\(^{2+}\) of the αS589C, -D, -N, and -A mutants is summarized in Fig. 4; the best fit of the data were obtained using the sum of two exponentials, consistent with two components of the ENaC block with a fast and a slow rate constant (Table 1). The data also show that increasing Cd\(^{2+}\) concentrations between 0.01 and 1 mM affect predominantly the time required to reach the equilibrium rather than the magnitude of current inhibition. From the extrapolation of the best fit of αSer-589 channel inhibition, we can predict that, at equilibrium, the half-maximal inhibition (\(IC_{50}\)) for Cd\(^{2+}\) is largely below 0.1 mM for the αS589C, -D, and -N mutants, suggesting a high affinity site for the Cd\(^{2+}\) binding site. However, the slow on-rate of the Cd\(^{2+}\) block points to the presence of important diffusional constraints in the αSer-589 mutants for Cd\(^{2+}\) ions to bind at its blocking site. Interestingly, the conserved αS589A substitution, which only slightly modifies the cation selectivity of the channel compared with the αS589C, -D, and -N mutants, shows the slowest on-rate of Cd\(^{2+}\) block.
asked whether differences may exist in the dissociation of Cd$^{2+}$ from its blocking site (off-rate) among the αS589C, αS589D, and αS589N mutants. The tracings in Fig. 6A compare the αS589C, -D, and -N mutants with respect to the recovery from amiloride and Cd$^{2+}$ block. The recovery from the channel inhibition by amiloride is rapid and nearly complete, whereas the recovery from Cd$^{2+}$ block is considerably slower and incomplete. The amount of $I_{Na}$ recovered 2 min after removal of external Cd$^{2+}$ was similar for the three αSer-589 mutants (Fig. 6C), indicating that coordination of Cd$^{2+}$ at position α589 is not responsible for its slow dissociation rate. In comparison, the recovery from blockade by Cd$^{2+}$ of the αS583C mutant is rapid and fully reversible (Fig. 6, B and C). These experiments further support our hypothesis that the mechanism of block of the αSer-589 mutants by Cd$^{2+}$ differs from the block of the S583C that involves direct coordination with the sulfhydryl side chain of the cysteine. Residues other than Cys or Asp introduced at position αSer-589 likely participate in the channel inhibition by external Cd$^{2+}$.

It can be argued that mutations at αSer-589 create a novel binding site that allows inhibition of $I_{Na}$ by Cd$^{2+}$ independently of the diffusion of this cation into the pore. However, if Cd$^{2+}$ has to bind within the pore in order to block the channel, this inhibition should be prevented by the pore blocker amiloride. To test this hypothesis we took advantage of the slow and partial reversibility of ENaC block by Cd$^{2+}$ and measured the recovery of $I_{Na}$ from channel inhibition by Cd$^{2+}$ when amiloride was simultaneously added to the external medium. Fig. 7A shows that the $I_{Na}$ of the αS589C, -D, and -N mutants exposed to 1 mM Cd$^{2+}$ in the presence of amiloride (10 μM) in the bath solution recovered rapidly and almost completely after removal of both blockers. These experiments demonstrate that amiloride prevents the irreversible inhibition of αSer-589 mutants by Cd$^{2+}$. These results are summarized in Fig. 7B in which it can be seen that the $I_{Na}$ recovered after ENaC inhibition by amiloride and Cd$^{2+}$ was identical to $I_{Na}$ after the same control period. The interpretation of these results is that upon binding to its receptor at the external entrance of the channel pore (see Fig. 1), amiloride prevents the access of Cd$^{2+}$ to its binding site located beyond that of amiloride. We conclude that the αS589N, as in the αS589C or -D mutants, Cd$^{2+}$ likely binds to cysteine resi-
ENaC Conduction Pore

A αS589C, β, γ

B αS589D, β, γ

C αS589N, β, γ

FIGURE 5. Time course of inhibition of ENaC current (I_{Na}) by Zn^{2+} in oocytes expressing αS589C, β, γ (A), αS589D, β, γ (B), or αS589N, β, γ (C) (closed symbols) compared with oocytes expressing ENaC wild-type (open symbols). Inhibition curves were obtained with Zn^{2+} concentrations of 0.1 mM (circle) or 1 mM (square). Data were best fitted by the sum of two exponentials, and results are given in Table 1. n = 4.

ENaC Conduction Pore

A αS589C, β, γ

B αS589D, β, γ

C αS589N, β, γ

FIGURE 6. Slow and partial recovery of ENaC currents from Cd^{2+} block. A, Oocytes were injected with αS589C, β, γ, αS589D, β, γ, or αS589N, β, γ. Removal of amiloride induced a large and reversible I_{Na}. The addition of 1 mM Cd^{2+} inhibited I_{Na} that slowly and partially recovered after Cd^{2+} removal. B, oocytes expressing αS583C, β, γ show a rapid and complete recovery of I_{Na} after inhibition by 1 mM Cd^{2+}. Scale, x-axis, 1 min; y-axis, Cys, 3 μA, Asp, 5 μA, Asn, 3 μA. C, normalized I_{Na} before, during, and 2 min after application of Cd^{2+} (1 mM) in the bath (n = 20).

ENaC Conduction Pore

A αS589C, β, γ

B αS589D, β, γ

C αS589N, β, γ

FIGURE 7. The nearly irreversible I_{Na} inhibition by Cd^{2+} is prevented by amiloride. A, oocytes expressing αS589C, β, γ, αS589D, β, γ, or αS589N, β, γ, were exposed to external Cd^{2+} (1 mM) in the presence of 10 μM amiloride; I_{Na} completely recovered after removal of Cd^{2+} and amiloride. Scale, x-axis, 1 min; y-axis, Cys, 3 μA, Asp, 5 μA, Asn, 3 μA. B, recovery of Cd^{2+} inhibition in the presence of amiloride (n = 6).

dues within the channel pore at a site downstream or deeper relative to the amiloride binding site.

For positively charged pore blockers binding within the transmembrane pore region, the large electric field across the membrane provides the energy to move the blocker along the channel pore. If Cd^{2+} binds within the transmembrane electric field, we expect the inhibition of αSer-589 ENaC mutants by Cd^{2+} to be dependent on voltage across the membrane. Fig. 8 shows the I_{Na} inhibition of the αS589N and αS589C mutants by 1 and 3 mM extracellular Cd^{2+} at transmembrane voltages ranging from −120 to 0 mV. Inhibition of both mutants by Cd^{2+}, arbitrarily determined 40 s after application, was clearly dependent on voltage, with a stronger inhibition at negative holding potentials. According to Woodhull’s formulation, the slope parameter z is equal to the product of the actual valence of the blocking ion (2 for Cd^{2+}) and the fraction of the electrical distance, δ, acting on Cd^{2+} at the binding site. The δ values of 0.34 ± 0.05 and 0.42 ± 0.05 calculated for the voltage dependence of Cd^{2+} block of αS589N and αS589C, respectively, represent apparent relative distances in the electric field, because they were not determined at equilibrium. Still our data clearly indicate that Cd^{2+} binds to a site within the transmembrane electric field that is indistinguishable for both mutants.

So far, our analysis of the current inhibition of ENaC αSer-589 mutants by Cd^{2+} reveals that this cation binds to a site in the channel pore located deeper than the amiloride binding site and that the contribution of the substituted residue at position α589 in coordinating Cd^{2+} ions to block the channel is not essential since the αS589N substitution also confers to the channel a sensitivity to Cd^{2+}. We further investigated this point by probing the αS589D mutant with external Ca^{2+} and looked...
for binding interactions between Ca²⁺ and the engineered aspartate side. The Ca²⁺ ions have an ionic radius comparable with Cd²⁺ or Zn²⁺. As shown in Fig. 9, Ca²⁺ at 10 mM in the external medium exerts only a modest inhibition of the αS589D mutant (~25% inhibition of Iₙa) compared with Cd²⁺ or Zn²⁺ (Figs. 4 and 5). Furthermore, in contrast to Cd²⁺, the weak block by Ca²⁺ was rapidly and completely reversible.

Taken together, our experimental evidence does not support the participation of the side chain of the amino acids at position αSer-589 in coordinating Cd²⁺, Zn²⁺, or Ca²⁺ ions, although such interaction cannot be firmly excluded in the absence of structural data. It seems more likely that the inhibition of the αS589C, -D, or -N substitution mutant by Cd²⁺ or Zn²⁺ is due to coordination with native cysteines in the internal pore of ENaC that become accessible to Cd²⁺ and Zn²⁺, due to the αSer-589 mutation in the channel selectivity filter.

There are a large number of cysteines in the α-, β-, and γENaC sequences that represent potential binding sites for Cd²⁺ ions in the internal pore of the channel. We have limited our analysis to conserved regions of the N terminus, the TM1 and TM2 transmembrane segments that involve 13 cysteine residues. We initially planned to substitute all the 13 cysteines, but we quickly realized that we were unable to generate a functional channel in the background of the αS589N mutation when more than 5 substituted cysteines were mutated in α-, β-, or γENaC subunits.

We have performed a first screen with different ENaC mutants in which native cysteines were substituted individually or in pairs in the αS589N mutant background. We have identified γCys-546 as a potential binding site for Cd²⁺. Recordings in Fig. 10, A–C, show the time course of Cd²⁺ inhibition of the S589C, -D, and -N mutants with the substitution of the native cysteine to serine at position Cys-546 in γENaC. When compared with the tracing in Fig. 2, the Iₙa inhibition by 0.1 mM Cd²⁺ was almost negligible for the αS589C/D/β, γC546S mutants and considerably reduced at 1 mM for the αS589N, β, γC546S. Fig. 10 (bottom in each panel) displays the effect of the γC546S mutation on the time course of Iₙa inhibition by Cd²⁺ of the αS589C, -D, -N mutants. At all the Cd²⁺ concentrations tested, the γC546S substitution decreases the magnitude of the Iₙa inhibition by Cd²⁺, but current inhibition persists at high Cd²⁺ concentrations. Such Iₙa inhibition in the presence of Cd²⁺ is expected for a channel mutant with a permeability to Cd²⁺ ions that is lower than for Na⁺ ions since the slower diffusion of Cd²⁺ along the pore will consequently reduce as well that of Na⁺.

We asked whether the lower sensitivity of the γC546S mutant to block by Cd²⁺ might be related to a faster dissociation rate from its binding site and tested the recovery of the αS589C/β, γC546S, αS589D/β, γC546S, and αS589N/β, γC546S mutants from Cd²⁺ block at millimolar concentrations. Iₙa recordings of the αS589C/β, γC546S, αS589D/β, γC546S, and αS589N/β, γC546S mutants in Fig. 11A show that, after the Cd²⁺-dependent decrease in Iₙa, removal of external Cd²⁺ allowed a fast and almost complete recovery of Iₙa. These experiments show that the γCys-546 is responsible for the almost irreversible trapping of Cd²⁺ within the pore that leads to the channel block.

**DISCUSSION**

The ENaC channel is insensitive to block by external Cd²⁺ but is highly sensitive to block by Cd²⁺ applied at micromolar concentrations from the intracellular side of the membrane (13). The cysteine substitution αS589C has two major consequences on channel function; first, it confers a block by externally applied Cd²⁺ or Zn²⁺ ions, and second, it changes the ion selectivity of the channel (11). Mutations at position αSer-589 have been shown to drastically change the ion selectivity of ENaC, allowing divalent cations to pass through the channel. Detectable currents carried by divalent cations such as Sr²⁺ or...
Ca\(^{2+}\) could be measured through \(\alpha\)S589C and \(\alpha\)S589D ENaC mutants (9).

The aim of this work was to understand the molecular mechanism of the block of the \(\alpha\)Ser-589 ENaC mutants by extracellular Cd\(^{2+}\) and to identify the amino acid residues coordinating Cd\(^{2+}\). We have hypothesized that external Cd\(^{2+}\) ions are unable to block wild-type ENaC essentially because the channel is impermeable to divalent cations. Rendering ENaC permeable to divalent cations by mutations at residues in the selectivity filter such as the \(\alpha\)Ser-589 should make the channel sensitive to block by external Cd\(^{2+}\) in a way similar to the ENaC block by internally applied Cd\(^{2+}\). This approach could be applied to identify the residues lining the conductive pore and therefore to better understand the structure of ENaC.

It has been recently proposed that the block of the \(\alpha\)S589C ENaC mutant by Cd\(^{2+}\) results from the coordination of Cd\(^{2+}\) with the sulfhydryl group of the 589 cysteine pointing toward the channel pore (11). The contribution of this cysteine site chain in coordinating Cd\(^{2+}\) ions seems questionable since alternative \(\alpha\)Ser-589 substitutions such as \(\alpha\)S589N render ENaC equally sensitive to block by Cd\(^{2+}\). Therefore, a sulfhydryl group at serine 589 is not necessary for Cd\(^{2+}\) block of the \(\alpha\)S589C mutant, and it is difficult on this basis to draw any conclusion about the side chain orientation of the residue at \(\alpha\)Ser-589 relative to the channel pore. The extremely weak inhibition of the \(\alpha\)S589D mutant by external Ca\(^{2+}\) at concentrations as high as 10 mM further supports the absence of interaction between divalent cations in the channel pore and the side chain residue at position \(\alpha\)Ser-589. It remains likely that, as for the KcsA K\(^{+}\) channel, the selectivity filter of ENaC is lined essentially by the backbone atoms of the G/SXS sequence as has been already proposed (10).

Our observations are consistent with a mechanism for Cd\(^{2+}\) blocking of the \(\alpha\)S589C mutant that involves a change in the selectivity filter allowing the access for external Cd\(^{2+}\) ions to a binding site within the internal pore. The block of \(\alpha\)S589C, \(\alpha\)S589D, and \(\alpha\)S589N by Cd\(^{2+}\) is characterized by a slow on-rate and an equally slow and partial recovery from blockade. These blocking kinetics are clearly different from those of \(\alpha\)S583C ENaC block by Cd\(^{2+}\). This latter mutant with the substitution located in the amiloride binding site at the outer entrance of the channel pore shows a fast and fully reversible current inhibition by submillimolar Cd\(^{2+}\) or Zn\(^{2+}\) consistent with the accessibility of the cysteine sulfhydryl group at position 583 to permeant ions. The slow on-rate of \(\alpha\)S589C block by Cd\(^{2+}\) rather suggests the presence of significant diffusional constraints for Cd\(^{2+}\) to reach a site within the channel pore where it binds nearly irreversibly and with high affinity. This is illustrated in our experiments by the fact that the concentration of external Cd\(^{2+}\) between 10 \(\mu\)M and 1 mM mainly affects the on-rate of block and has relatively little effect on the maximal current inhibition.

Our experimental data provide little evidence for a direct interaction between Cd\(^{2+}\) and the sulfhydryl group of the cysteine at position \(\alpha\)589. The question remains where does Cd\(^{2+}\) bind? The voltage dependence of the channel block by Cd\(^{2+}\)

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**FIGURE 10. Effect of the ENaC \(\gamma\)C546S mutation on the Cd\(^{2+}\) block.** Effect of \(\gamma\)C546S on the Cd\(^{2+}\) block in the background of \(\alpha\)S589C (A, \(n = 10\)), \(\alpha\)S589D (B, \(n = 5\)), or \(\alpha\)S589N (C, \(n = 10\)). Top, representative recordings of Cd\(^{2+}\) block. Bottom, direct comparison of the time course of 0.1 mM (circle), 1 mM (square), or 10 mM (triangle) Cd\(^{2+}\) inhibition of \(\alpha\)Ser-589 mutants alone (closed symbols) or together with the \(\gamma\)C546S mutation (open symbols).
The αSer-589 ENaC mutants provide an interesting model to identify the cysteine residues that coordinate Cd\(^{2+}\) ions along the ion permeation pathway. Unfortunately, in our search for cysteines interacting with Cd\(^{2+}\), we were limited by the low amiloride-sensitive current of the cysteine substitution ENaC mutants performed in the background of the αSer-589 mutations. It was thus difficult to obtain a complete picture of the cysteines lining the internal pore of the channel. However, we identified Cys-546 in the TM2 of the γENaC subunit as a residue that contributes to the channel block by Cd\(^{2+}\). First, the γC546S mutation decreases the apparent affinity for Cd\(^{2+}\) of a site that is responsible for the fast component of the channel inhibition. Second, the γC546S substitution makes the channel block by Cd\(^{2+}\) rapidly reversible. Such increase in the off-rate of Cd\(^{2+}\) blocking kinetics represents a strong argument supporting both a direct participation of the γCys-546 in the coordination of Cd\(^{2+}\) ion and the orientation of the sulfhydryl group of γCys-546 toward the ion permeation pathway. The γCys-546 residue is likely the first accessible site for external Cd\(^{2+}\) ions, and Cd\(^{2+}\) binding to this site results in a channel block. The slower component of the Cd\(^{2+}\) block seems unaffected by γCys-546 mutation, suggesting the presence of a deeper and less accessible site for binding Cd\(^{2+}\) along the internal part of the channel pore. The γCys-546 may not be the only residue involved in the coordination with Cd\(^{2+}\) ions. The identification of additional cysteine partners involved in binding interactions with Cd\(^{2+}\) ions requires the substitution of these cysteines in the background of the αSer-589 and γCys-546 mutations. Unfortunately, these ENaC constructs did not express measurable amiloride-sensitive currents.

Even though several structural models have been proposed, the structure of the ion channel pore of the members of the ENaC/degenerin family has yet not been elucidated (16, 18). Functional analysis of voltage-dependent blocking of ENaC by impermeant cations supports a funnel-like structure of the external conduction pore of ENaC that can accommodate amiloride in its outer mouth (position 583 in the rat ENaC sequence) and then narrowing down to the selectivity filter (G/SXS sequence, Fig. 1) (19). The primary sequence of this pore region (Ser-583–Ser-589 in rat αENaC, Fig. 1) does not allow reliable secondary structure predictions. However, the second transmembrane domain starting at the conserved Val residue Val-590 conforms to a highly probable α helix (Fig. 1) (8). The side chain orientation of the amino acid residues lining the pore region has clearly been demonstrated for the residues involved in the binding interactions with amiloride, i.e. Ser-583 in the αENaC (Fig. 1) and the corresponding glycine residues in the β and γ ENaC subunits (17). The thiol side chains of cysteine substitutions at positions Leu-584, Trp-585, Phe-586, and Ser-588 in the αENaC (Fig. 1) and γENaC (Fig. 1) and γENaC (Fig. 1) are suggested to be involved in the binding interactions with Cd\(^{2+}\) ions.

Our experiments support the view that the substitutions at position Ser-589 in the αENaC subunit result in steric modifications of the channel pore that allow externally applied Cd\(^{2+}\) to reach a binding site normally inaccessible within the internal pore of ENaC located beyond the selectivity filter. Wild-type ENaC is blocked by micromolar concentrations of Cd\(^{2+}\) applied from the intracellular side, and this block shares two important characteristics with the block of α589C mutant by extracellular Cd\(^{2+}\): it displays both an affinity in the micromolar range and is slowly and only partially reversible. The steric modifications resulting from αSer-589 mutations affect the ionic selectivity of ENaC, making the channel more permeable to K\(^+\) and Ca\(^{2+}\). It has not been possible to show that the αSer-589 mutants are permeable to Cd\(^{2+}\) or Zn\(^{2+}\) ions, since both divalent cations block the channel, but it remains very likely that the steric modifications in the narrowest part of ENaC, due to αSer-589 mutation, allow Cd\(^{2+}\) ions to pass the selectivity filter and to bind cysteine residues located along the channel pore. In the case of wild-type ENaC, these binding sites for Cd\(^{2+}\) are only accessible from the intracellular side.
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αS89C mutant (11). Thus, from this experimental evidence, the αSer-583 appears to be the only residue in the pore region sequence having its side chain facing the ion conduction pore and interacting with ions present in the pore lumen. Amino acid substitutions of the corresponding glycine in β (Gly-525) and γ (Gly-537) ENaC subunits suggest orientations of these residues similar to Ser-583 in α subunit (17, 20).

The second transmembrane α helix likely starts with residue Val-590, according to model predictions of the αENaC subunit (Fig. 1) (8). The Cys-546 in the γENaC interacts with permeant Cd$^{2+}$ ions in the background of the αSer-589 mutation, providing strong evidence that the thiol side chain is facing the ion conduction pore. It is therefore likely that γCys-546 is accessible to permeant ions after passing successively through the external pore and the selectivity filter. This represents the first evidence for the contribution of the second transmembrane segment to the internal pore lining.

A previous cysteine accessibility scan performed on ASIC1a using methanethiosulfonates applied from the intracellular side of the channel revealed that cysteines introduced at several positions corresponding in the γENaC sequence to Val-547, Ile-548, and Ile-550, as well as others located more distally in the TM2, were inaccessible to MTSET, i.e. did not interact with internal MTSET to block the channel (14). Thus, according to the presently available experimental evidence, it seems that only the first residues in the proximal part of the TM2 corresponding to position γCys-546 participate in the pore lining. In the Kv1.2, the distal part of TM2 forms the tip of “inverted tepee” architecture that corresponds to the intracellular end of the conduction pore (12).

Recent evidence suggests that the intracellular start of the TM1 and part of the amino terminus of ASIC and ENaC channels participate to the inner pore structure (13, 14). We can therefore conclude that the ion conduction pore of ENaC/ASIC channels does not conform to the general structural feature of the potassium channels.

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