A Gating Mutation in the Internal Pore of ASIC1a*

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Using a substituted cysteine accessibility scan, we have investigated the structures that form the internal pore of the acid-sensing ion channel 1a. We have identified the amino acid residues Ala-22, Ile-33, and Phe-34 in the amino terminus and Arg-43 in the first transmembrane helix, which when mutated into cysteine, were modified by intracellular application of MTSET, resulting in channel inhibition. The inhibition of the R43C mutant by internal MTSET requires opening of the channel. In addition, binding of Cd\(^{2+}\) ions to R43C slows the channel inactivation. This indicates that the first transmembrane helix undergoes conformational changes during channel inactivation. The effect of Cd\(^{2+}\) on R43C can be obtained with Cd\(^{2+}\) applied at either the extracellular or the intracellular side, indicating that R43C is located in the channel pore. The block of the A22C, I33C, and F34C mutants by MTSET suggests that these residues in the amino terminus of the channel also participate to the internal pore.

The epithelial sodium channel (ENaC)\(^2\) and the acid-sensing ion channels (ASICS) in mammals are members of the recently identified ENaC/degenerin family of voltage-insensitive channels (1). The epithelial sodium channel mediates Na\(^+\) transport in renal and airway-tight epithelia (2). The ASICS are expressed in the central and peripheral nervous system and are possibly involved in nociception, learning, or mechanosensation (3).

ENaCs and ASICS are likely formed by four homologous subunits. The membrane topology of the channel subunits predicts an amino and a carboxyl terminus facing the inside of the cell, the presence of two transmembrane segments, and a large extracellular loop (4–7). The structural basis of ENaC/ASIC function remains poorly understood. Amino acid residues in the extracellular loop of ENaC preceding the second transmembrane domain (TM2) bind the pore blocker amiloride (8, 9). Based on previous studies on the interaction between permeant Na\(^+\) ions and pore blockers of different sizes, it has been proposed that the external pore vestibule of ENaC, where amiloride binds, narrows down to the selectivity filter allowing a Na\(^+\) or Li\(^+\) ion to selectively permeate the channel (10). This model is supported by the identification of residues at the external end of ENaC TM2 near the amiloride binding site, which are important for maintaining its high selectivity for Na\(^+\) (11–15). Beyond the external selectivity filter in TM2, the channel structures lining the ion permeation pathway that are accessible from the cytosol have not yet been identified.

In this study, we have used the homomultimeric ASIC1a as a model to investigate the structure of the internal ASIC/ENaC pore. Recently, we have observed that multiple cysteine residues in the amino terminus of ENaC subunits are at least, in part, responsible for the sensitivity of ENaCs to inhibition by intracellular sulfhydryl-modifying agents (16). This suggests that the amino terminus participates in ENaC gating and/or constitutes the internal pore of the channel. In the present study, we have investigated the role of the amino terminus and the start of the first transmembrane segment in controlling the ion flux through ASIC1a. We have found that cysteine substitutions in the amino terminus and in TM1 are modified by intracellularly applied sulfhydryl reagents, thereby inhibiting ASIC1a currents. We provide evidence that the cytosolic part of the TM1 is located at the internal channel pore and undergoes conformational changes during channel gating.

EXPERIMENTAL PROCEDURES

ASIC1a Expression—Complementary cDNA of the human ASIC1a (17) was subcloned in the pSDEasy cloning vector for in vitro transcription and expression in Xenopus oocytes. Stage V and VI Xenopus oocytes were injected with 5 ng of cRNA encoding hASIC1a.

Electrophysiology—Electrophysiological measurements were performed 24–36 h after oocyte injection with ASIC cRNA. Macroscopic ASIC currents (\(I_{ASIC}\)) were elicited every 30 s by rapid changes in extracellular pH from 7.4 to 6.0 and were measured using either the two-electrode voltage clamp for whole-cell currents or the cut-open oocyte technique when intracellular perfusion was needed. The two-electrode voltage clamp measurements were performed as described previously (9). The bathing solution contained (in mm) NaCl 120, MgCl\(_2\) 2, HEPES-H\(^{+}\) 10 mm, adjusted to pH 7.5 with NaOH. Changes in extracellular pH were achieved using the same bathing solution with MES-H\(^{+}\) 10 mm buffered at pH 6.0. The cut-open configuration of the Xenopus oocyte allows the recording of macroscopic ASIC currents while continuously perfusing the inside (2 \(\mu\)l min\(^{-1}\)) and the outside (>8 ml min\(^{-1}\)) of the oocytes (16). A microperfusion pipette, in which two thin capillaries (Microfil, World Precision instruments) had been inserted, was used for the intracellular perfusion and served as an intracellular electrode potential measurement. The intracellular solution contained (in mm) potassium gluconate 90, KCl 10, sodium gluconate 2, MgCl\(_2\) 1, BAPTA 0.2, HEPES-N-ethyl-N-glycine 10, adjusted to pH 7.35. Methanethiosulfonates or Cd\(^{2+}\) (1 mm) were added to the solution. The holding potential was −100 mV. The extracellular solution corresponded to the bathing solution in the two-electrode voltage clamp experiments.

Site-directed Mutagenesis—Introduction of cysteine residues in the ASIC1a sequence was performed as described previously for ENaC mutagenesis (9). The presence of the mutation in the ASIC1a cDNA was
verified by sequencing. cRNA was synthesized in vitro for injection into the oocytes.

Chemicals—The methanethiosulfonate (MTS) reagents purchased from Toronto Research Chemicals (Toronto, Canada) were MTSET, MTSEE, and MTSET-PtrEA. Stock solutions with MTS reagents were prepared in Me2SO and diluted at least 100-fold, immediately before use, into the intracellular solution.

RESULTS

Typical recordings of ASIC1a wild type (Fig. 1) in internally perfused oocytes (cut-open configuration) show that ASIC currents (IASIC) elicited by short external pH changes (2 s duration) from 7.4 to 6.0, were stable for several minutes. The perfusion of intracellular methanethiosulfonates (MTSEA, MTSET, MTSET-PtrEA at 1 mM) did not affect the magnitude of IASIC. Similar recordings were obtained in the presence of MTSET in the external medium (data not shown). The insensitivity of ASIC1a to inhibition by intracellular and extracellular MTSET reagents indicates that the cysteine residues Cys-49, Cys-59, and Cys-61 in the first transmembrane segment (TM1) of ASIC1a wt (see Fig. 2A) are not modified by MTSET or, if modified, do not inhibit ASIC currents. Data reported on an ortholog of ENaC, the peptide-gated Na\(^+\) channel (FaNaCh), using a systematic cysteine substitution in TM1 showed 50–60% inhibition of the FaNaCh current by external MTS reagents (18). It was concluded that the TM1 of FaNaCh is lining the external pore of the channel. We could not confirm these findings on ASIC1a.

To test the solvent accessibility of the amino terminus of ASIC1a, cysteine residues were introduced by site-directed mutagenesis from position Ile-18 in the amino terminus to Trp-46 in TM1, and the mutants were expressed in Xenopus oocytes (Fig. 2A). Fig. 2B illustrates typical IASIC expressed by mutants of ASIC1a wt with cysteine substituted at positions A22C and R43C. For these mutants, IASIC decreased after the application of internal MTSET (1 mM), indicating that cysteine residues at position Ala-22 and Arg-43 are modified. R43C was similarly inhibited by the larger methanethiosulfonates MTSET-PtrEA 1 mM (73.9 ± 4.4% inhibition of IASIC after 2 min), indicating that R43C is accessible to large molecules. Fig. 3 summarizes the results obtained from the systematic cysteine substitution and analysis of modification by intracellular MTSET of residues Ile-18 to Trp-46. Substitutions at positions His-28, Gly-29, and His-32 were not investigated, because the corresponding mutations in ENaC result in channel loss of function (19). A few other cysteine substitutions, T26C, S35C, E37C, and W46C resulted in a complete loss of function. The substituted cysteine residues at positions Ala-22, Ile-33, Phe-34, or Arg-43 are modified by intracellular MTSET resulting in ASIC1a inhibition. The A22C, I33C, F34C, or R43C mutants were unresponsive to external MTSET at 2.5 mM (data not shown). The R38C mutant showed a weak but significant inhibition by internal MTSET.

The cysteine residue at position Ala-22 in the ASIC1a sequence corresponds to the conserved cysteine residues αCys-88, βCys-30, γCys-33 in rat ENaC subunit sequences (see Fig. 2A) that have been shown to be involved in the block of ENaC by intracellular MTS reagents (16). Thus, ENaC inhibition by an internal MTS reagent can be reproduced in ASIC1a with the A22C substitution. Additional cysteine residues in ENaC corresponding to Ser-35 and Leu-30 in ASIC1a also participate in ENaC inhibition by MTS reagents. The ASIC S35C mutant was not functional, but the adjacent cysteine substitutions I33C and F34C resulted in IASIC inhibition by internal MTSET. By contrast to ENaC, exposure of L30C to MTSET did not result in current inhibition. Taken together, these results indicate that cysteine residues flanking the HG motif in both ENaC and ASIC1a are accessible from the cytosol by MTS reagents and result in channel inhibition upon modification by sulfhydryl reagents.

State-dependent Accessibility—We next determined whether the substituted cysteine residues at position Ala-22, Ile-33, or Arg-43 were preferentially modified by MTS reagents in the open or closed conformation of the channel. We have compared the time course of ASIC1a inhibition by MTSET during repeated pH pulses applied every 0.5 min after maintaining the channel in the resting state for 2 min. It is clear from the recordings (Fig. 4A) and from the time course of IASIC inhibition (Fig. 4B) that maintaining ASIC1aR43C in the resting (closed) state in the presence of internal MTSET (1 mM) prevents ASIC block and delays the time course of channel inhibition (Fig. 4B). These data are consistent with a reduced accessibility of R43C to internal MTSET when the channel is closed and indicate that the TM1 undergoes conformational changes associated with channel gating. Comparison of the current inhibition of the A22C, I33C, and R43C ASIC1a mutants using the same protocol shows that keeping the channel in the resting state (Fig. 4C) did not affect the magnitude of inhibition of the A22C and I33C mutants. This indicates that the modifications of A22C and I33C by contrast to R43C are state-independent, suggesting that neither Ala-22 nor Ile-33 undergo conformational changes during channel gating that affect their accessibility.

The Internal Pore of ASIC1a

FIGURE 1. Current recordings of ASIC1a wild type. IASIC was elicited by short extracellular pH drops from 7.4 to 6.0. MTSEA or MTSET, or MTSET-PtrEA were continuously perfused inside the oocyte (filled bars).

FIGURE 2. Sensitivity of ASIC1a mutants to internal MTSET. A sequence alignment of the amino terminus and the first transmembrane helix (TM1) of ASIC1a and ENaC α, β, and γ subunits. Dark lines represent putative α helices predicted from the amino acid sequence. • are residues mutated into cysteine, and ▽ are cysteine residues Cys-49, Cys-59, and Cys-61 that were mutated in Ala, Val, and Ser, respectively. ASIC current (IASIC) recorded from A22C and R43C mutants before and after the internal perfusion of MTSET at 1 mM.
Effects of Internal Cd\textsuperscript{2+} Ions—Cadmium binds to sulfhydryl groups and is of a similar size as Na\textsuperscript{+} ions. Internal Cd\textsuperscript{2+} (1 mM) inhibits ASIC1a wt, and this inhibition is completely abolished by the C61S mutation in the TM1 (Fig. 5A), suggesting that C61 is accessible to intracellular Cd\textsuperscript{2+} ions. By contrast to MTSET reagents, internal Cd\textsuperscript{2+} did not inhibit \textit{i}_{\text{ASC}} of the R43C mutant more than wt. The most striking effect of Cd\textsuperscript{2+} on the R43C was a broadening of the \textit{i}_{\text{ASC}} transient caused by slowing of open channel inactivation. These data are summarized in Fig. 5, A–D, showing the effects of Cd\textsuperscript{2+} at 3 and 10 mM applied externally.

Accessibility from the Extracellular Side—ASIC1a is permeable to divalent cations such as Ca\textsuperscript{2+} (20). It is likely that, because of its smaller ionic radius, Cd\textsuperscript{2+} also permeates the channel. We hypothesized that, if the substituted cysteine R43C or Cys-61 is located along the ion permeation pathway, it should also bind Cd\textsuperscript{2+} from the extracellular side.

Extracellular Cd\textsuperscript{2+} (3 and 10 mM) decreases the \textit{i}_{\text{ASC}} expressed by ASIC1a wt and R43C mutants, suggesting that the size of the sulfhydril-modifying molecule is important for channel block. Second, the state-dependent block by MTSET and the effect of Cd\textsuperscript{2+} on the R43C mutant provides evidence that TM1 undergoes conformational changes during channel inactivation.

FIGURE 3. Inhibitory effect of internal MTSET on wild type and cysteine substitution mutants of ASIC1a. Relative \textit{i}_{\text{ASC}} (%) was determined from the ratio of \textit{i}_{\text{ASC}} with internal MTSET (1 mM) to \textit{i}_{\text{ASC}} without MTSET after 2 min. ND denotes ASIC1a mutants with loss of function. * denotes \( p < 0.01 \). Each bar represents the mean of 4–10 experiments with mean ± S.D.

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Extracellular Cd\textsuperscript{2+} (3 and 10 mM) decreases the \textit{i}_{\text{ASC}} current expressed by ASIC1a wt (71.9 ± 5.5% and 95.8 ± 1.1% inhibition, respectively (Fig. 6, A and C). The \textit{IC}_{50} for \textit{i}_{\text{ASC}} inhibition by external Cd\textsuperscript{2+} ions, as determined from concentration/inhibition curves, was similar for ASIC1a wt (0.37 ± 0.005 mM), for the R43C (0.46 ± 0.048 mM), and for the C49A/C59V/C61S triple mutant in the TM1 (0.42 ± 0.045 mM). Thus, both extracellular and intracellular Cd\textsuperscript{2+} inhibits ASIC1a. However, C61S mutation prevents the ASIC1a inhibition by internal Cd\textsuperscript{2+} but has no effect on ASIC1a inhibition by external Cd\textsuperscript{2+}. This indicates that external Cd\textsuperscript{2+} either binds to Cys-61 without impairing the ion flux.
through the pore or cannot access the Cys-61 residue from the external side. This observation is consistent with the absence of ASIC1a inhibition by external MTS reagents and do not favor a location of Cys-61 residue in the TM1 along the ion permeation pathway.

In contrast to ASIC1a wt, external Cd\(^{2+}\) reversibly slowed the current inactivation of the R43C mutant (Fig. 6, B, D, and E), resulting in a 3-fold increase in \(\tau\). Thus, intracellular and extracellular Cd\(^{2+}\) have similar effects on the time constant of \(I_{ASIC}\) inactivation of the R43C mutant, indicating that R43C binds Cd\(^{2+}\) applied from either side of the membrane. This provides strong evidence that Arg-43 is located in the ion permeation pathway. Together, our data show that TM1 not only moves during channel gating but that part of it also lines the internal pore.

**DISCUSSION**

Our results have identified amino acid residues in the amino terminus and the TM1 of ASIC1a that, when substituted with cysteine, are modified by intracellular MTS reagents, resulting in channel inhibition.

The amino-terminal sequence of ASIC1a comprising Phe-21 to Ile-33 shows a high degree of homology between the members of the ENaC/degenerin family with notably a completely conserved HG motif (see Fig. 2A). According to the current models of the membrane topology of ENaCs/degenerins, the amino terminus up to the first transmembrane segment TM1 of ASIC is facing the cytosolic side of the membrane. The functional importance of this amino-terminal domain has been first evidenced by a mutation in the HG motif of ENaC leading to a genetic form of a salt-losing nephropathy (pseudohypoaldosteronism type-1) (21). The ENaCs with mutations of the HG motif show altered gating properties characterized by abnormally long closures and short channel openings. More recently it has been shown that the high sensitivity of ENaC to intracellular sulfhydryl reagents and oxidizing agents can be attributed to conserved cysteine residues in the amino terminus of ENaC subunits (16). The mutants A22C, I33C, and F34C in the amino terminus of ASIC1a reproduce the ENaC sensitivity to inhibition by internal MTS reagents, indicating that the functional domains in the amino terminus are conserved among ENaCs and ASICs.

Our data do not allow us to conclude that the inhibition of the A22C, I33C, or F34C mutants by internal MTS reagents results from a block of the channel pore. The amino terminus of ENaC has been shown to play a role in channel gating (21). We have been unable to show that the permeant divalent cation Cd\(^{2+}\) binds to A22C or I33C when applied from either side of the membrane, as expected if A22C or I33C would be located along the ion permeation pathway. Thus, we are still lacking direct evidence that A22C and I33C are lining the internal pore of ASIC. Our results also show that A22C and I33C do not undergo conformational changes that modify their accessibility to intracellular MTS reagents. Thus, we have no good evidence that A22C and I33C are part of the gating machinery of the channel. It should be mentioned that mutations in the ASIC2 isoform at positions corresponding to Ala-20, Phe-21, and Thr-26 in the ASIC1a sequence (Fig. 2A) change the ion selectivity of the ASIC2, suggesting that this region of the amino terminus contributes to the channel pore structure (22). Clearly, the molecular mechanism underlying the inhibition of the A22C and I33C mutants by MTS reagents still remains to be elucidated to determine the contribution of these residues in the channel gating and/or in the permeation properties of the channel.

It is surprising that the A22C or I33C ASIC1a mutants are insensitive to blocking by millimolar concentrations of intracellular Cd\(^{2+}\), whereas ENaC with cysteine residues at positions corresponding to Ala-22 and Ser-35 is highly sensitive to blocking by internal Cd\(^{2+}\) (see Fig. 2A) (16). It is possible that channel inhibition by internal Cd\(^{2+}\) requires the binding interaction of Cd\(^{2+}\) with several cysteine residues, such as Ala-22 and Ser-35 or other cysteines present in ENaC but absent in ASIC1a sequences.

The location of R43C in the ion permeation pathway is supported by the fact that R43C is accessible to Cd\(^{2+}\) from both the extracellular and intracellular sides. Furthermore, the modification of R43C by internal MTS reagents indicates that this Arg residue lines the internal channel pore. Together with the state-dependent accessibility of R43C to MTS reagents, this suggests the presence of an internal gate that hinders the access of ions to the internal pore of the channel. Binding of Cd\(^{2+}\) to R43C may act as a “foot in the door” delaying channel inactivation.

Regarding the Cys-49, Cys-59, and Cys-61 located in the TM1, we do not have evidence that these residues line the channel pore, even though Cys-61 seems to play a role in the ASIC1a inhibition and to be accessible by internal Cd\(^{2+}\).

**The Channel Pore**—Fig. 7 represents a linear model of the transmembrane segments of ASIC and ENaC that summarizes the structures known to be involved in the ion permeation pathway. The external ion channel pore is made of a short putative \(\alpha\) helix that contains a site (degenerin site) that, when modified by external MTS in the open conformation of the channel, freezes the channel in the open state (23, 24). Further downstream, the binding site for the external pore blocker amiloride has been identified in ENaC (8, 9). The first residues in TM2 of ENaC (selectivity filter) are accessible to external Cd\(^{2+}\) and are important for maintaining the ion selectivity and the ionic conductance of the channel (11–15). There is little evidence supporting a contribution of the distal part of TM2 in the internal pore lining. We have generated and tested the substitution mutants V450C, L451C, L453C, F454C, Y458C, R467C, R468C, G469C, and K470C in the cytosolic end of TM2. Exposure to 1 mM internal MTSET did not result in current inhibition in any of these mutants, suggesting that TM2 does not participate in the internal mouth of the channel. Our data represent the first demonstration that TM1 is involved in the pore lining and participates in channel gating; this study identifies the amino terminus of the ENaCs/ASICs as an important functional domain accessible from the cytosol and as controlling the ion flux through the channel.
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