Intracellular Thiol-mediated Modulation of Epithelial Sodium Channel Activity*

Stephan Kellenberger, Ivan Gautschi, Yvan Pfister, and Laurent Schild‡

From the Department of Pharmacology and Toxicology, University of Lausanne, rue du Bugnon 27, Lausanne CH-1001, Switzerland

The epithelial sodium channel ENaC is physiologically important in the kidney for the regulation of the extracellular fluid volume, and in the lungs for the maintenance of the appropriate airway surface liquid volume that lines the pulmonary epithelium. Besides the regulation of ENaC by hormones, intracellular factors such as Na\(^+\) ions, pH, or Ca\(^{2+}\) are responsible for fast adaptive responses of ENaC activity to changes in the intracellular milieu. In this study, we show that ENaC is rapidly and reversibly inhibited by internal sulfhydryl-reactive molecules such as methanethiosulfonate derivatives of different sizes, the metal cations Cd\(^{2+}\) and Zn\(^{2+}\), or copper(II) phenanthroline, a mild oxidizing agent that promotes the formation of disulfide bonds. At the single channel level, these agents applied intracellularly induce the appearance of long channel closures, suggesting an effect on ENaC gating. The intracellular reducing agent dithiothreitol fully reverses the rundown of ENaC activity in inside-out patches. Our observations suggest that changes in intracellular redox potential modulate ENaC activity and may regulate ENaC-mediated Na\(^+\) transport in epithelia. Finally, substitution experiments reveal that multiple cysteine residues in the amino and carboxyl termini of ENaC subunits are responsible for this thiol-mediated inhibition of ENaC.

The highly selective and amiloride-sensitive epithelial sodium channel ENaC\(^1\) expressed at the apical membrane of tight epithelia represents the main pathway for sodium absorption to maintain the level and the composition of the air-surface liquid (2).

Hormonal and nonhormonal factors control ENaC activity in epithelia. In the distal nephron, the cell surface expression of ENaC is regulated by aldosterone and vasopressin. This hormonal control of ENaC activity at the cell surface involves intracellular cascades of events that are not yet fully understood (3). Nonhormonal stimuli such as intracellular pH, Ca\(^{2+}\) or Na\(^+\) ions achieve a rapid regulation of ENaC activity, to control the influx of Na\(^+\) ions into the cells (4).

ENaC belongs to a heterogeneous family of ion channels which contains channels regulated by hormones such as ENaC or acid-sensing ion channels that are gated by external protons (5). These channels are heteromeric channels composed of subunits with two putative transmembrane segments (TM1 and TM2) separated by a large extracellular loop. They share with other genetically unrelated channels, such as K\(^+\) channels, a similar membrane topology with the amino and carboxyl termini of the subunits facing the intracellular side of the membrane. These α, β, and γ subunits of ENaC are arranged pseudosymmetrically around the channel pore so that all subunits participate in specific ENaC functions such as channel block by amiloride, ionic permeability, and selectivity (6–8). To achieve high ionic selectivity like K\(^+\) channels, ENaC is likely composed by a limited number of subunits; several evidences support a four-subunit stoichiometry composed of 2α, 1β, and 1γ ENaC subunits (6). The binding site for amiloride has been located in a short segment of the extracellular loop preceding the second transmembrane segment (TM2), which likely forms the outer entrance of the channel. An external modulatory site for ENaC activity is located upstream from the amiloride binding site in the ENaC sequence (9, 10). At the extracellular 5’ start of the TM2 segment a short stretch of 3 amino acids is important to prevent larger cations such as K\(^{+}\) or ammonium ions from passing through the channel and likely forms the external narrow ionic selectivity filter in ENaC (8, 11).

The extracellular loop of ENaC contains domains rich in cysteine residues that are highly conserved among the members of the ENaC degenerin family. These extracellular cysteine residues of ENaC are likely to be involved in the formation of disulfide bonds important for the proper folding of the protein (12). Cysteine residues are also found in the transmembrane segments and in the amino and carboxyl termini of α, β, and γ ENaC subunits and are potentially accessible from the intracellular milieu (Fig. 1). The amino termini of αβγ ENaC subunit sequences contain together 8 cysteine residues, and 5 of them are conserved among species (from amphibian to human). They flank a highly conserved HG motif that plays an important role in ENaC gating (13). The role of these intracel-
compartments and provides an electrical isolation between the extracellular (upper) and intracellular (lower) compartments, by clamping the electrical voltage of the guard compartment to the same voltage as the external (upper) compartment. The voltage clamp was performed using a Dagan cut-open oocyte voltage clamp apparatus (Dagan Corporation, Minneapolis, MN; model CA-1 high performance oocyte clamp).

The extracellular (upper) compartment was perfused at a rate of ~8 ml/min with an extracellular solution containing 80 mM sodium gluconate, 10 mM tetraethylammonium-Cl, 5 mM BaCl₂, 1 mM MgCl₂, 0.5 mM CaCl₂, 10 mM Hepes-N-methyl-d-glucamine, pH 7.5, with or without 10 μM amiloride. ENaC current (Iₜot) was defined as the difference between the current flowing through the portion of the oocyte membrane exposed to the upper compartment, in the absence and presence of 10 μM amiloride. During the experiment, the intracellular compartment was kept at a holding potential of ~100 mV. The intracellular electrode was a glass pipette made of a single barreled glass capillary (Kwik-Fil Borosilicate TW100–4, World Precision Instruments) in which two thin capillaries (Microfil, World Precision Instruments) had been inserted far enough that their tips were close to the pipette tip. This allowed rapid changes of intracellular solutions with or without thiol reagents (perfusion rate 2 μl/min). The intracellular solution contained 90 mM potassium gluconate, 10 mM KCl, 2 mM sodium gluconate, 1 mM MgCl₂, 200 μM BAPTA, 10 mM Hepes-N-methyl-d-glucamine, pH 7.35.

The effects of extracellular sulfenate (MTS) reagents, C2S₄, on oxidizing conditions on single channel currents were performed in the inside-out configuration of the patch clamp technique, as described previously (16). The bath solution in patch clamp experiments was similar as the intracellular solution in cut-open oocyte measurements and contained 90 mM potassium gluconate, 10 mM KCl, 2 mM sodium gluconate, 1 mM MgCl₂, 10 μM BAPTA, 200 μM TCEP, 10 mM Hepes-N-methyl-d-glucamine, pH 7.35. Changing of extracellular solutions in patch clamp experiments was done using the Rapid Solution Changer RSC-200 (Biologic, Claix, France). The pipette solution had the same composition as the extracellular solution in cut-open experiments with either Na⁺ or Li⁺ ions as charge carriers through ENaC.

In patch clamp experiments, currents were recorded with a List EPC-9 patch clamp amplifier, using a PC-based data acquisition system (List, HEKA Electronic, Lambrecht/Pfalz, Germany) and filtered at 1000 Hz for single channel analysis. Single channel openings were detected by standard half-amplitude threshold analysis, using TAC 4.09 (Bruxton Corporation, Seattle, WA). The channel number was estimated from the maximum number of overlapping openings. NPₜ was calculated from the TACFIT event table. Averaged data are presented as the mean ± S.E.

Macroscopic whole cell currents were recorded using the two-electrode voltage clamp technique at a holding potential of ~100 mV. Currents were recorded with a Dagan TEV-200 amplifier (Minneapolis, MN) equipped with two bath electrodes. The standard bath solution contained 110 mM NaCl, 1.8 mM CaCl₂, 10 mM Hepes-NaOH, pH 7.35. Site-directed Mutagenesis and Expression in X. laevis Oocytes—Site-directed mutagenesis was performed on rat α₄β₇γ ENaC cDNAs as described previously (7). Mutant cDNAs were controlled by sequencing (Synergene, Zurich, Switzerland).

The cysteine residues in the amino terminus of ENaC subunits were replaced by alanine in case introduction of serine might potentially introduce phosphorylation sites in the ENaC sequence; cysteine residues in the transmembrane segments were replaced by serine residues. The different mutant constructs of α₄β₇γ rat ENaC subunits are listed in Table 1. Each mutant construct was expressed either with the two homolog wild type or in combination with other mutant ENaC subunits.

Chemicals—Copper(II) phenanthroline (Cu(Phen)₂) was made from a 5 mM 1,10-phenanthroline stock solution in dry ethanol and a 1.5 mM cupric sulfate stock solution in water, diluted to the final concentration of 15 μM 1,10-phenanthroline and 50 μM cupric sulfate in the bathing solution. Amiloride and other chemicals were purchased from Sigma. MTS reagents were from Toronto Research Chemicals (Toronto, Canada), and TCEP was from Calbiochem. The following MTS reagents were used: MTSEA, MTSET, MTSES, MTSEA-biotin, and MTS-PhEA. Stock solutions of MTS reagents were prepared in dimethyl sulfoxide and diluted at least 100-fold, immediately before use, into the intracellular solution. Intracellular application of 100-fold diluted dimethyl sulfoxide alone did not affect ENaC function (data not shown).

RESULTS

Despite the presence of numerous cysteine residues in the extracellular loop ENaC expressed in Xenopus oocytes is poorly sensitive to inhibition by extracellular MTS reagents; a 20–
50% inhibition of ENaC current by external MTSEA has been reported (17, 18). Because MTSEA can cross membranes to a certain degree (19), we have determined in preliminary experiments the effects of external MTSEA and MTSET on ENaC currents in *Xenopus* oocytes. Oocytes were incubated with these MTS reagents for 15 min before the current measurements. The ENaC-mediated amiloride-sensitive current measured with the two-electrode voltage clamp was not significantly different in oocytes incubated with or without 1 mM external MTSEA, respectively, when applied from the intracellular side of the cell membrane.

**ENaC Inhibition by Intracellular Thiol Reagents**—To investigate the effects of MTS reagents applied from the cytosolic side on ENaC activity, we used the so-called cut-open oocyte technique, which permits intracellular perfusion of the oocyte (14, 15). Oocytes were mounted in a specially designed chamber, and a fine glass pipette was positioned into the oocyte from the bottom of the chamber, allowing internal perfusion of the oocyte with a continuously flowing solution. The activity of ENaC was measured as the current sensitive to 10 μM amiloride applied to the external side of the oocyte membrane (see “Experimental Procedures”). Typical recordings of the ENaC current measured across the plasma membrane of an oocyte perfused from both the cytosolic and extracellular sides are shown in Fig. 2A. Removing amiloride from the extracellular solution induces a robust inward current that usually reaches hundreds of nanoamps in magnitude and remains stable for several minutes. The inward current returns to base line after readdition of external amiloride (Fig. 2A, upper left panel). To test the effects of intracellular thiol reagents on ENaC activity, external amiloride was first removed to induce an inward ENaC current, then 0.1 mM MTSEA or analogous reagents were applied intracellularly. Immediately after the start of MTSEA perfusion, the inward ENaC current started to decrease exponentially over a period of 2–3 min. The residual current in the presence of MTSEA returned to base line after readdition of external amiloride, indicating that the current inhibited by intracellular MTSEA represents a major fraction of the ENaC-mediated amiloride-sensitive Na⁺ current (IₐNa). Similar inhibitory effects were observed on ENaC currents after the intracellular application of other MTS reagents (MTSET, MTS-PTeEA, or MTSEA-biotin). Fig. 2B summarizes the inhibitory effects of MTS reagents different in size: concentrations of 0.1 mM for MTSET and MTSEA and 0.5 mM for the larger MTS-PTeEA and MTSEA-biotin lead to a similar maximal inhibition after 4 min and a comparable time course of the ENaC current decrease. The rate constant (kₑₐ) calculated from the exponential fit of the time course of IₐNa inhibition was 4,198 μ⁻¹ min⁻¹ for MTSEA, 3,786 μ⁻¹ min⁻¹ for MTSET, and 0.488 μ⁻¹ min⁻¹ for MTSEAPₐₐ, respectively. This difference in kₑₐ for the large MTS reagents might be explained by a slower diffusion rate in the perfused oocyte. 

**TABLE I**

| Mutations     | αENaC       | βENaC       | γENaC       |
|---------------|-------------|-------------|-------------|
| Single        | C88A        | C30A        | C33A        |
|               | C101A       | C43A        | C41A        |
|               | C118S       | C534S       | C71S        |
|               | C557S       | C30A/C43A   | C33A/C41A   |
|               | C557S       | C30A/C534S  | C33A/C546S  |
|               | 10G/C30A    | C546S       | C546S/C546S |
| Double        | C88A/C101A  | C101A/C118S | C33A/C41A/C71S |
|               | C88A/C101A/C118S | C308/C43A/C534S | C33A/C41A/C546S |
| Triple        | C37S/C88A/C101A | C101A/C118S | C33A/C41A/C71S |
|               | C88A/C101A/C118S | C557S/P646stop | C546S/C546S |
| Deletions     | C645S/P646stop | C557S/R564stop | W574stop |

**Fig. 2. Effects of different methanethiosulfonates on ENaC currents.** A, representative traces showing IₐNa in cut-open oocytes expressing rat ENaC (rENaC) and IₐNa inhibition after intracellular 0.1 mM MTSEA, 0.1 mM MTSET, and 0.5 mM MTS-PTeEA. B, time course of IₐNa inhibition by different methanethiosulfonates, 0.1 mM MTSEA (○), 0.1 mM MTSET (▲), 0.5 mM MTSEA-biotin (△), and 0.5 mM MTS-PTeEA (□). The corresponding filled symbols represent control IₐNa measurements in the absence of intracellular MTS compounds. Each point represents the mean ± S.E. of 5–12 measurements. The dotted lines represent a fit to an exponential equation (γ = e⁻ᵏₑₐt), which gave rate constants k for a IₐNa decrement of 0.419 min⁻¹ in the presence of MTSEA, 0.379 min⁻¹ for MTSET, 0.478 min⁻¹ for MTSEA-biotin, and 0.488 min⁻¹ for MTS-PTeEA. C, effects of 0.1 mM MTSEA on ENaC current in oocytes expressing the *Xenopus* ENaC (xENaC) or the human α₇ ENaC (hENaC) orthologs.
It should be mentioned that the presence of 10 mM dithiothreitol (DTT) in the perfusion solution prevented the inhibitory effects of MTSEA on $I_{Na}$ but did not completely reverse the inhibitory effect (data not shown). These experiments indicate that cysteine residues on ENaC subunits that are accessible to large MTS molecules from the cytosolic side inhibit ENaC activity upon covalent modification. Finally we tested whether the sensitivity of ENaC to intracellular sulfhydryl-reactive molecules is a property of rat ENaC only or whether it is shared by other ENaC orthologs. Fig. 2C shows that in oocytes expressing X. laevis or human ENaC, MTSEA similarly inhibited the ENaC-mediated current, indicating that inhibition by intracellular sulfhydryl reagent is a general characteristic of the ENaC.

**Divalent Cations**—Transition metal cations such as Cd$^{2+}$ or Zn$^{2+}$ can modulate the activity of a number of voltage- or ligand-gated channels by coordinating reduced thiol groups of accessible cysteine residues. We postulated that internal Cd$^{2+}$ and Zn$^{2+}$ have similar inhibitory effects on ENaC activity as MTS reagents if free thiol groups of cysteine residues are involved in channel block. Fig. 3A shows representative recordings of $I_{Na}$ in oocytes internally perfused with different concentrations of Cd$^{2+}$ or Zn$^{2+}$, using protocols similar to those for MTS reagents in Fig. 2. It should be mentioned that stable current recordings in the cut-open oocyte require the presence of Ca$^{2+}$ chelators such as BAPTA in the intracellular solution to avoid activation of Ca$^{2+}$-dependent ion conductances. In the presence of 200 $\mu$M BAPTA, 500 $\mu$M intracellular Cd$^{2+}$ or 500 $\mu$M Zn$^{2+}$ inhibited the ENaC current, whereas lower concentrations (100 $\mu$M) where without effects, likely because of the low concentration of free Cd$^{2+}$ or Zn$^{2+}$. The precise IC$_{50}$ for ENaC inhibition by Cd$^{2+}$ or Zn$^{2+}$ was thus difficult to assess precisely. In a few successful and stable recordings without intracellular BAPTA, concentrations as low as 10 $\mu$M Cd$^{2+}$ were effective in inhibiting ENaC (75.6 $\pm$ 4.9% of $I_{Na}$ inhibition after 2 min, $n = 4$). The time course of $I_{Na}$ inhibition seemed slightly more rapid than for MTS reagents, and ENaC inhibition was almost complete 3 min after the beginning of the Zn$^{2+}$ or Cd$^{2+}$ application (Fig. 3B). As for the effect of MTSEA in the cut-open oocyte, recovery from Cd$^{2+}$ inhibition was not complete after the addition of 10 mM EDTA in the internal perfusate (data not shown). These results, together with the effects of MTS reagents, strongly suggest that modification of reduced thiol groups of intracellular cysteine residues by methanethiosulfonates or transition metals rapidly inhibits ENaC activity.

**ENaC Block at the Single Channel Level**—Macroscopic ENaC current recorded in cut-open oocytes is a function of the number of functional ENaC molecules expressed at the cell surface ($N$), the ENaC unitary ionic conductance ($g$), and the open probability ($P_o$) of the channel. In principle, a change in any of these three components of the macroscopic ENaC current could account for the effect of thiol reagents. To differentiate between changes in single channel conductance and in channel $P_o$, single channel activity was recorded in the inside-out patch clamp configuration. As illustrated in Fig. 4, rapid application of 50 $\mu$M MTSET to the intracellular side of an inside-out patch containing more than 10 active ENaC channels. The application of 50 $\mu$M MTSET to the intracellular side of the membrane induced a rapid inhibition of ENaC activity without changes in the magnitude of the single channel current. B, ENaC recording in an inside-out patch starts with 50 $\mu$M MTSET in the bath facing the cytosolic side of the membrane patch. After removal of MTSET and addition of 10 mM DTT in the bath, typical ENaC activity could be recorded after ~10 s.
ENaC blocking reaction by MTSET was 4270 ± 521 m s⁻¹ (n = 9) and faster in inside-out membrane patches compared with that measured in cut-open oocytes. This is consistent with the presence of significant diffusional constraints for MTS reagents in the internally perfused oocytes.

Table II summarizes the effects of MTSET on ENaC open probability: MTSET inhibits more than 95% of ENaC current; inhibition is not complete, and rare and rapid channel reopenings can be observed in the presence of MTSET, as shown in the recording of Fig. 4A. During the application of MTSET the magnitude of single channel current remained unchanged (0.929 ± 0.103 pA before MTSET and 0.852 ± 0.174 pA after MTSET, measured at −100 mV with Li⁺ ions as charge carrier).

The inhibitory effect of MTSET on ENaC was not spontaneously reversible. To provide further evidence that cysteine modifications by sulfhydryl-reactive molecules are responsible for ENaC inhibition, we tested whether the thiol-reducing agent DTT could reverse the effects of MTSET. The recording in Fig. 4B starts in the presence of 50 μM MTSET in the bathing solution, and ENaC activity is almost nonexistent except rare and fast openings. Removal of MTSET and the subsequent addition of 10 mM DTT resulted after ~10 s in the appearance of typical ENaC activity and revealed the presence of at least four active ENaC channels in the patch. The data in Table II show that DTT significantly reverses the effect of MTSET; however, the reducing effect of DTT did not allow a complete recovery of ENaC activity. This can be the result of an incomplete dissociation of the MTSET from the cysteine residues in the presence of DTT; we cannot exclude a spontaneous rundown of ENaC activity over the duration of the recording which could at least in part explain this partial recovery.

The inhibitory effect of Cd²⁺ on ENaC current was also investigated at the single channel level. The application of intracellular Cd²⁺ at micromolar concentrations (20 μM Cd²⁺, together with 10 μM BAPTA in the solution, see “Experimental Procedures”) rapidly closes the channel. As shown in Fig. 5A, ENaC inhibition by 20 μM intracellular Cd²⁺ is characterized by the appearance of long channel closures over a time period of several seconds, without significant changes in the single channel current amplitude (0.28 ± 0.007 pA without and 0.27 ± 0.01 pA in the presence of 20 μM Cd²⁺ at −50 mV measured with Na⁺ ions, n = 4). This effect of intracellular Cd²⁺ on ENaC resembles that of covalent modification of cysteine residues by MTSET; in the presence of Cd²⁺, the recording shows no evidence for channel reopenings or fast transitions between open and closed states, as would be expected for a rapidly reversible interaction between the ligand and its coordination site. This observation is consistent with a slow dissociation rate of Cd²⁺ from its binding site. This is supported further by the slow recovery of ENaC activity after Cd²⁺ removal, even in the presence of 2 mM EDTA (Fig. 5B). The average time delay for reappearance of a stable ENaC activity was 28.8 ± 4.6 s (n = 5), and the recovery was only partial (Table II). As control, 20 μM intracellular Ni²⁺ or 20 μM Ba²⁺ was without effects on ENaC activity in inside-out patches (n = 2, data not shown). Thus, the effects of intracellular sulfhydryl reagents or Cd²⁺ on ENaC current are consistent with a thiol-mediated inhibitory effect on ENaC that, at the single channel level, is characterized by the appearance of long channel closures.

Redox Modulation of ENaC Activity—The discovery of the inhibitory effects of intracellular cysteine modifications on ENaC activity prompted us to investigate whether the formation of disulfide bonds between intracellular cysteine residues under mild intracellular oxidizing conditions has any effect on ENaC activity. We used Cu(Phe)₃ as a mild thiol-oxidizing agent applied intracellularly to inside-out patches. As shown in Fig. 6A, intracellular application of 15:50 μM Cu(Phe)₃ induces a rapid reduction in ENaC activity over a time period of 10–15 s, which contrasts with the stable channel activity during the control period. Again, the decrease in ENaC activity, characterized by the appearance of long closures similar to those observed with intracellular MTSET or Cd²⁺, suggests a common mechanism for the three inhibitors. As shown in Table II, 90% of ENaC activity was inhibited by Cu(Phe)₃ and, as for Cd²⁺ or MTSET, a low but significant residual ENaC activity was detected in the presence of the oxidizing reagent (NP = 0.56 ± 0.16). The similarity of the ENaC inhibition at the single channel level by MTSET, Cd²⁺, and Cu(Phe)₃ suggests a common mechanism.
It is unlikely that the inhibitory effect of Cu(Phe)_3 on ENaC activity is the result of free Cu^{2+} ions in the bathing solution because of the high stability of the Cu(Phe)_3 complex and also because of the presence of 10 μM BAPTA in the bathing solution. The concentration of free Cu^{2+} in the bathing solution calculated, using a stability constant of the Cu(Phe)_3 complex of 2 × 10^6 M⁻¹, is around 5 nM (20). In inside-out patches, the addition of 0.2 μM Cu^{2+} (without phenanthroline) to the bathing solution had no effect on ENaC activity, as measured from the N_Po (2.65 ± 1.26 n = 4 in the absence and 2.67 ± 1.35, n = 4 after addition of Cu^{2+}). These experiments suggest that under mild oxidizing conditions the formation of intracellular disulfide bonds between at least one pair of cysteine residues results in inhibition of ENaC activity.

This inhibitory effect of Cu(Phe)_3 on ENaC activity was not reversed by simple removal of the oxidizing reagent. However, addition of reducing agents such as DTT (10 mM) at least partially reversed the inhibitory effect of Cu(Phe)_3 (Table II). In inside-out patches the redox state of the bathing solution is usually poorly controlled, and we asked whether the reducing agent DTT was able to reactivate ENaC after a spontaneous rundown of activity. In these experiments made in inside-out configuration, we investigated ENaC channels that had a significant rundown of activity in the absence of reducing agents in the bathing solution. A significant rundown was defined as a loss of more than 90% of ENaC activity over the first 40 s of recording. Fig. 6B shows a recording starting with inactive ENaC channels in a patch that had spontaneously disappeared after 30 s. The application of DTT to the cytosolic side of this patch restored ENaC activity which then remained stable. Table II shows the effects of DTT on channels with spontaneous rundown, and gives the N_Po values measured at the beginning of ENaC recordings, at the end of the rundown, and after application of 10 mM intracellular DTT. The results indicate that the reducing agent DTT was able to reactivate ENaC fully and demonstrate that changes in the intracellular redox potential are able to modulate ENaC activity. We were unable to modulate ENaC activity with treatment of the oocytes with NO donors or by direct intracellular application of these agents in the cut-open oocyte.

Cysteine Mutagenesis—To understand further the mechanisms underlying the inhibition of ENaC by thiol-reactive agents, we used site-directed mutagenesis to identify the critical cysteine residues. As already pointed out, 20 cysteine residues in the αβγ ENaC sequence are located in the predicted transmembrane segments or cytosolic amino- and carboxyl-terminal parts of the channel and are potentially facing the intracellular side (Fig. 1). We have generated single or multiple cysteine substitution mutants that target all of the potential cysteines in the ENaC sequence which could be involved in the thiol-mediated inhibition of ENaC. We found that none of these α or β or γ ENaC mutants listed in Table I, with expressed with the two homologous wild type subunits, modified significantly the ENaC sensitivity to intracellular block by MTSEA or Cd^{2+}, indicating the participation of multiple cysteines in this process.

We have then combined the different αβγ ENaC constructs listed in Table I to generate mutants lacking all major cysteines (see also Fig. 1) in the amino terminus (αC37S/C38A/C101A, βC106G/C30A/C43A, γC33A/C41A) (A); an ENaC substitution mutant of cysteines in TM1 αC118S, βC61S/C64S, γC71S (B); an ENaC substitution mutant of cysteines in the carboxyl terminus αC645S/P646stop, βC575S/R564stop, γC565S/P564stop, βC106G/C30S/C43A/C557S/P646stop, γC33A/C41A (E).

Fig. 7. Effects of cysteine substitutions on the inhibition of ENaC-mediated current by intracellular MTSEA. Traces on the left side of the figure were obtained under conditions of internal perfusion with control solution; traces on the right side were obtained with internal perfusion of 0.1 mM MTSEA. Currents were measured in a cut-open oocyte expressing an ENaC substitution mutant of cysteines in the amino terminus αC37S/C38A/C101A, βC106G/C30A/C43A, γC33A/C41A (A); an ENaC substitution mutant of cysteines in TM1 αC118S, βC61S/C64S, γC71S (B); an ENaC substitution mutant of cysteines in TM1 αC118S, βC534S, γC542S/C546S (C); an ENaC substitution mutant of cysteines in the carboxyl terminus αC645S/P646stop, βC575S/R564stop, γC565S/P564stop, βC106G/C30S/C43A/C557S/P646stop, γC33A/C41A (E).
in the absence of intracellular MTSEA reagents nor the channel sensitivity to block by MTSEA, as shown by the robust inhibition of ENaC current after internal application of MTSEA (Fig. 7, B, C, and D). The substitutions of the cysteine residues in the amino terminus of αβγ ENaC subunits resulted in a slight but significant rundown of ENaC activity during the intracellular perfusion of the oocyte in the absence of MTSEA (Fig. 7A). However, this ENaC mutant lacking cysteines in the amino terminus is still sensitive to intracellular MTSEA. Thus substitutions of the cysteine residues either in the amino terminus, or the TM1, or the TM2, or in the carboxyl terminus of all three αβγ ENaC subunits were not sufficient to abolish the sensitivity of the channel to intracellular MTSEA. Finally we generated an ENaC mutant lacking cysteines in the amino terminus and carboxyl terminus of the αβγ ENaC subunits by coexpressing the αC88A/C101A/C118A/C645S/P646stop, βC10G/C30S/C43A/C557S/P564stop, γC534S/C557S/C61S/C64S, E, carboxy-terminal mutant αC645S/P646stop, βC557S/R564stop, γF, amino- and carboxyl-terminal mutant αC88A/C101A/C118A/C645S/P646stop, βC10G/C30S/C43A/C557S/P564stop, γC33A/C41A.

in the amino terminus and carboxyl terminus of ENaC, the amino and carboxyl termini. The precise contribution of each of the 13 cysteine residues located in the amino terminus and carboxyl terminus, in ENaC inhibition by MTSEA, remains to be established.

**DISCUSSION**

In this study, we have shown for the first time that ENaC can be blocked from the cytosolic side by sulphhydryl-reactive MTS reagents and by the transition metal cations Cd\(^{2+}\) and Zn\(^{2+}\) reacting with thiol groups of cysteine residues. ENaC inhibition by these sulphhydryl-reacting agents can be reversed by DTT or by EDTA. Furthermore, oxidizing agents that promote disulphide bond formation inhibit ENaC activity when applied intracellularly, whereas intracellular reducing agents such as DTT reverse spontaneous rundown of channel activity. Cysteine substitution experiments indicate that several cysteine residues located in the amino and carboxyl terminus of ENaC subunits, but not in the two transmembrane domains, are responsible for the ENaC inhibition by the thiol-reactive agent MTSEA.

**Complex Mechanism of ENaC Inhibition by Thiol Reagents**—MTSs, Cd\(^{2+}\), and Cu(Phe)\(_n\), reacting with intracellular cysteine thiol groups, inhibit ENaC activity by inducing channel closures. No significant changes in single channel current amplitudes were observed in the presence of the intracellular thiol reagents. The three reagents, Cd\(^{2+}\), MTSET, and Cu(Phe)\(_n\), have similar effects on single channel activity, suggesting that they affect the same channel function via modifications of intracellular cysteine residues. Two general mechanisms can be proposed to account for the ENaC inhibition by the intracellular thiol reagents. The target cysteine residues are located within the ion permeation pathway, and the coordination of Cd\(^{2+}\), MTS reagents, or oxidizing agents with the thiol groups of cysteines may impair the ion flow through the channel pore. Alternatively, these reactions occur outside the ion permeation pathway in a region important for channel gating and lead to long and sustained channel closures. Our data do not allow us to distinguish firmly between these two possibilities. However, the observation that the inhibition of ENaC activity is independent of the size of the thiol-coordinating agent suggests that this inhibition is not the result of a plugging of the ion channel pore by the ligand. Rather this suggests that the cysteine residues that coordinate the sulphhydryl-reacting agents are accessible to large internal molecules such as MTS-biotin, at a site important for the modulation of channel activity.

The ENaC block by Cd\(^{2+}\) involves a strong binding interaction with intracellular cysteine residues, as shown in inside-out patches by the slow rate of Cd\(^{2+}\) dissociation and the slow recovery of channel activity after Cd\(^{2+}\) removal. Similar observations were done on the Shaker K\(^+\) mutant channel with cysteine residues introduced in the S6 transmembrane segment that line the internal pore of the channel: such mutant K\(^+\) channels are highly sensitive to inhibition by intracellular Cd\(^{2+}\), which is essentially irreversible even in the presence of chelators of Cd\(^{2+}\) such as EGTA (21). Such a tight interaction with Cd\(^{2+}\) involves in this Shaker K\(^+\) mutant, multiple cysteine residues in the S6 segment of the different subunits, as well as the trapping of Cd\(^{2+}\) inside the internal pore of the K\(^+\) channel. The comparable affinities for Cd\(^{2+}\) block of ENaC and this K\(^+\) mutant channel are consistent with multiple intracellular cysteine residues involved in the coordination of intracellular Cd\(^{2+}\) ions. However, we have not been able to provide evidence for Cd\(^{2+}\) trapping inside the channel pore during channel closures, mainly because ENaC openings or closures cannot be easily controlled, as in the Shaker K\(^+\) channel by the voltage.

The participation of multiple cysteine residues in thiol-mediated ENaC inhibition is further supported by the inhibitory effect of Cu(Phe)\(_n\), which promotes the formation of disulphide...
bonds between pairs of closely localized thiol groups. Finally, the fact that substitutions of a single cysteine were not sufficient to abolish ENaC block by Cd$^{2+}$ or MTSs strongly support the participation of several intracellular cysteine residues in the thiol-mediated modulation of ENaC activity.

Intracellular Cysteine Switches and ENaC Function—Substitution of the 4 cysteine residues in the TM1 or the 3 cysteine residues in the TM2 of the $\alpha\beta\gamma$ ENaC subunits does not affect ENaC function nor its sensitivity to inhibition by thiol reagents. We can conclude that the cysteine residues in the transmembrane $\alpha$ helices of ENaC are not primarily involved in the channel block by internal thiol reagents. These two transmembrane helices TM1 and TM2 have been proposed as structures lining the pore of ENaC channel homologs (18, 22). Our data show that the cysteines in TM1 (cC118, $\beta$C61/C64, $\gamma$C71) and TM2 (bC534, $\gamma$C542/C546) are not facing the internal channel pore at a site where MTS-reactive agents can have access. The contribution of the other residues in the TM1 and TM2 in the internal pore lining of ENaC remains to be demonstrated.

Substitutions of cysteine residues in the amino terminus of ENaC subunits significantly decrease the fraction of MTS-reactive ENaC current, but the channel still remains sensitive to this thiol-reactive agent. Complete resistance to current inhibition by MTSEA is obtained after substitution and deletions of the cysteines located in the amino terminus and in the carboxyl terminus. These amino and carboxyl termini of $\alpha\beta\gamma$ contain 13 cysteines, and among these putative targets for sulphydryl-reactive agents, the precise numbers and positions of the cysteines involved in ENaC inhibition remain to be determined.

The inhibition of ENaC by intracellular sulphydryl-reactive agents is a general phenomenon conserved among the Xenopus, rat, and human orthologs. Among the members of the ENaC-degenerin family, the ENaC subunits are the only channel subunits having multiple conserved cysteine residues in the proximity of the highly conserved HG motif in the cytosolic amino terminus. Secondary structure predictions of the amino terminus of ENaC are compatible with the presence of an amphipathic $\alpha$ helix of 10 amino acids which is separated from the start of TM1 by a linker that includes the highly conserved HG motif (see Fig. 1). The cysteine residues conserved among species in each ENaC subunit (cC88/C101, $\gamma$C33/C41) are predicted to be located in the 3′-end of the amphipathic $\alpha$ helix and in the linker preceding the first TM1 segment containing the HG motif. These cysteines represent good candidates for ENaC inhibition upon chemical modifications by sulphydryl reagents.

The conserved HG motif is the target for a missense mutation in $\beta$ENaC causing a mild form of pseudohyposaldosteronism type 1 (23). Alanine substitutions of the conserved His-94, Gly-95, and the neighboring Arg-98 residues in $\alpha$ENaC decrease channel activity at the cell surface (13). Together with single channel analysis of the Arg-98 ENaC mutant, it was proposed that the segment encompassing the residues His-94 to Arg-98 was involved in the modulation of channel gating (24). The proximity of the conserved amino-terminal cysteines (cC88, aC101, $\beta$C30, $\gamma$C33, $\gamma$C41) to the intracellular HG motif suggests the possibility that covalent modifications of these cysteines by MTS reagents might freeze the channel in the closed conformation.

The cysteines in the carboxyl terminus are not conserved among species, although sequence alignment is difficult in this region of the ENaC subunit sequences. It is clear from our data that the substitutions of the oC645 and $\beta$C557 together with truncations at positions oP646 and $\beta$R564 do not affect per se ENaC sensitivity to inhibition by MTSEA, unless all of the amino-terminally located cysteines are replaced. It is this combination of cysteine substitutions in the amino and carboxyl termini which confers resistance to ENaC. Thus our data indicate that, in addition to the role of the amino terminus, the carboxyl terminus is important for ENaC function and likely contributes either in the internal pore of the channel and/or in channel gating.

Physiological Relevance—The functional and physiological relevances of intracellular cysteine residues that modulate ENaC activity remain to be established. ENaC activity decreases in the presence of intracellular oxidizing agents and can be restored in the presence of the reducing agent DTT. This suggests the possibility that these cysteine residues may link ENaC activity to the redox state of the cell, as ENaC is expected to be switched off by this mechanism under intracellular oxidative stress.

Cysteine residues have important regulatory roles at active sites of different ion channels. Modulation of ion channel activity can be observed after reaction of cysteine sulphydryl groups with thiol reagents, transition metals, or nitric oxide. The NMDA receptor activity is modulated by Zn$^{2+}$, nitric oxide, and MTS reagents, which tend to decrease NMDA-evoked currents (25, 26). At least seven extracellular cysteine residues are involved in this thiol-mediated regulation of the NMDA receptor activity. In addition to this complexity, redox or S-nitrosylation reactions can compete for the same thiol group to modulate NMDA channel activity.

Cyclic nucleotide-gated channels are activated by NO, and site-directed mutagenesis studies have identified a cysteine residue located close to the cytosolic cGMP binding site which is responsible for the channel S-nitrosylation and changes in channel gating (27). ENaC activity is clearly not modulated by NO, neither are the intracellular cysteine residues of ENaC part of a consensus sequences for protein nitrosylation.

The physiological relevance of the modulation of ENaC by intracellular cysteine residues remains to be established. The main question remains as to whether changes in the redox potential within the cell are sufficiently large to influence the reduced state of cysteine thiol groups in the amino terminus of ENaC. It has been shown that the changes in the redox potential can modulate the gating kinetics of the cystic fibrosis conductance regulator (CFTR). Reducing conditions accelerate both a subconductance opening rate and the closing rate of the channel, whereas oxidizing conditions slow channel gating (28, 29). In these studies intracellular Cd$^{2+}$ also prolonged the opening bursts of the CFTR. Furthermore, changes in the redox ratio of electron carriers such as pyridine nucleotides NAD(P)$^+/NAD(P)$H modulate the cAMP-dependent Cl$^-$ conductance mediated by the CFTR (30). A shift in the redox ratio to more oxidized NAD(P)$^+$ results in an increase in the CFTR whole cell current. Finally, application of the sulphydryl-modifying agent N-ethylmaleimide to cytosolic domains of the CFTR stimulates channel activity (31). As with ENaC, the CFTR contains numerous intracellular cysteine residues, and evidence supports the contribution of multiple cysteine residues located in the second nucleotide binding domain and in the regulatory domain, in the modulation of CFTR activity by redox and sulphydryl reagents.

Taking these observations reported in the literature on CFTR and our data on ENaC together, it appears that changes in the redox conditions can modulate differentially the CFTR and ENaC. ENaC and CFTR are important in the lungs where the height of the airway surface liquid lining the apical side of the respiratory epithelium is tightly regulated and involves a balance between sodium absorption via ENaC and chloride secretion by the CFTR (2). Our study showing that ENaC...
activity can be modulated by oxidizing and reducing agents provides a potential molecular mechanism linking the redox status of the airway epithelial cells exposed to different conditions of anoxia and hyperoxia, with secretion or absorption of Na\(^+\) and Cl\(^-\) in the airway epithelium.

Acknowledgments—We thank J.-D. Horisberger and B. C. Rossier for helpful discussions and comments on the manuscript.

REFERENCES

1. Lifton, R. P., Gharavi, A. G., and Geller, D. S. (2001) Cell 104, 545–556
2. Boucher, R. C. (2004) Eur. Respir. J. 23, 146–158
3. Verrey, F., Hummler, E., Schild, L., and Rossier, B. C. (2001) in The Kidney (Seldin, D.W., and Giebisch, G., eds) pp. 1441–1472, Lippincott Williams & Wilkins, Philadelphia
4. Garty, H., and Palmer, L. G. (1997) Physiol. Rev. 77, 359–396
5. Kellenberger, S., and Schild, L. (2002) Physiol. Rev. 82, 735–767
6. Firsov, D., Gautschi, I., Merillat, A. M., Rossier, B. C., and Schild, L. (1998) EMBO J. 17, 344–352
7. Schild, L., Schneeberger, E., Gautschi, I., and Firsov, D. (1997) J. Gen. Physiol. 109, 15–26
8. Kellenberger, S., Hoffmann-Pochon, N., Gautschi, I., Schneeberger, E., and Schild, L. (1999) J. Gen. Physiol. 114, 13–30
9. Kellenberger, S., Gautschi, I., and Schild, L. (2002) J. Physiol. (Lond.) 543, 413–424
10. Snyder, P. M., Bucher, D. B., and Olson, D. R. (2000) J. Gen. Physiol. 116, 781–790
11. Kellenberger, S., Gautschi, I., and Schild, L. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 4170–4175
12. Firsov, D., Robert-Nicoud, M., Gruender, S., Schild, L., and Rossier, B. C. (1999) J. Biol. Chem. 274, 2743–2749
13. Grunder, S., Firsov, D., Chang, S. S., Jaeger, N. F., Gautschi, I., Schild, L., Lifton, R. P., and Rossier, B. C. (1997) EMBO J. 16, 899–907
14. Abriel, H., and Horisberger, J. D. (1999) J. Physiol. (Lond.) 516, 31–43
15. Tagialatela, M., Toro, L., and Stefani, E. (1992) Biophys. J. 61, 78–82
16. Kellenberger, S., Gautschi, I., and Schild, L. (2003) Mol. Pharmacol. 64, 848–856
17. Snyder, P. M., Cheng, C., Prince, L. S., Rogers, J. C., and Welsh, M. J. (1998) J. Biol. Chem. 273, 661–684
18. Sheng, S. H., Li, J. Q., Mcmurt, K. A., Kieber-Emmons, T., and Kleyman, T. R. (2001) J. Biol. Chem. 276, 1263–1234
19. Holmgren, M., Liu, Y., Xu, Y., and Yellen, G. (1996) Neuropharmacology 35, 797–804
20. Neale, E. J., Elliott, D. J., Hunter, M., and Sivaprasadarao, A. (2003) J. Biol. Chem. 278, 29079–29085
21. Liu, Y., Holmgren, M., Furrman, M. E., and Yellen, G. (1997) Neuron 19, 175–184
22. Poet, M., Taue, M., Lingenue, G., Canoe, P., Poujoel, P., Lazdunski, M., and Couillen, L. (2001) EMBO J. 20, 5505–5502
23. Chang, S. S., Grunder, S., Hanukoglu, A., Rosler, A., Mathew, P. M., Hanukoglu, I., Schild, L., Lu, Y., Shalmkets, R. A., Nelson-Williams, C., Rossier, B. C., and Lifton, R. P. (1996) Nat. Genet. 12, 248–253
24. Grunder, S., Jaeger, N. F., Gautschi, I., Schild, L., and Rossier, B. C. (1999) Pflugers Arch. Eur. J. Physiol. 438, 709–715
25. Das, S., Sasaki, Y. F., Rothe, T., Premkumar, L. S., Takasu, M., Crandall, J. E., Dikkes, P., Conner, D. A., Rayudu, P. V., Cheung, W., Chen, H. V., Lipton, S., and Nakanishi, N. (1998) Nature 373, 377–381
26. Lipton, S. A., Choi, Y. B., Takahashi, H., Zhang, D. I., Li, W., Godzik, A., and Bankston, L. A. (2002) Trends Neurosci. 25, 474–480
27. Brouillet, M. C., and Firestein, S. (1999) Neuron 18, 951–958
28. Harrington, M. A., Gunderson, K. L., and Kopito, R. R. (1999) J. Biol. Chem. 274, 27536–27544
29. Harrington, M. A., and Kopito, R. R. (2002) Biophys. J. 82, 1278–1292
30. Stutts, M. J., Gabriel, S. E., Price, E. M., Sarkadi, B., Olsen, J. C., and Boucher, R. C. (1994) J. Biol. Chem. 269, 8667–8674
31. Cotten, J. F., and Welsh, M. J. (1997) J. Biol. Chem. 272, 25617–25622
Intracellular Thiol-mediated Modulation of Epithelial Sodium Channel Activity
Stephan Kellenberger, Ivan Gautschi, Yvan Pfister and Laurent Schild

J. Biol. Chem. 2005, 280:7739-7747.
doi: 10.1074/jbc.M409955200 originally published online December 28, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M409955200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 30 references, 16 of which can be accessed free at http://www.jbc.org/content/280/9/7739.full.html#ref-list-1