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Inhibition of Lung Fluid Clearance and Epithelial Na^+ Channels by Chlorine, Hypochlorous Acid, and Chloramines*

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We investigated the mechanisms by which chlorine (Cl_2) and its reactive byproducts inhibit Na^+-dependent alveolar fluid clearance (AFC) *in vivo* and the activity of amiloride-sensitive epithelial Na^+ channels (ENaC) by measuring AFC in mice exposed to Cl_2 (0–500 ppm for 30 min) and Na^+ and amiloride-sensitive currents (I_{Na} and I_{amil}, respectively) across *Xenopus* oocytes expressing human α-, β-, and γ-ENaC incubated with HOCl (1–2000 μM). Both Cl_2 and HOCl-derived products decreased AFC in mice and whole cell and single channel I_{Na} in a dose-dependent manner; these effects were counteracted by serine proteases. Mass spectrometry analysis of the oocyte recording medium identified organic chloramines formed by the interaction of HOCl with HEPES (used as an extracellular buffer). In addition, chloramines formed by the interaction of HOCl with taurine or glycine decreased I_{Na} in a similar fashion. Preincubation of oocytes with serine proteases prevented the decrease of I_{Na} by HOCl, whereas perfusion of oocytes with a synthetic 51-mer peptide corresponding to the putative furin and plasmin cleaving segment in the γ-ENaC subunit restored the ability of HOCl to inhibit I_{Na}. Finally, I_{Na} of oocytes expressing wild type α- and γ-ENaC and a mutant form of βENaC (S520K), known to result in ENaC channels locked in the open position, were not altered by HOCl. We concluded that HOCl and its reactive intermediates (such as organic chloramines) inhibit ENaC by affecting channel gating, which could be relieved by proteases cleavage.

The balance of fluid covering the respiratory and alveolar epithelia is determined in part by the ability of these cells to transport sodium (Na^+) and chloride (Cl^-) ions in a vectorial fashion. Active Na^+ reabsorption across lung epithelia requires the coordinated entry of Na^+ ions through cation- and Na^+-selective amiloride-sensitive channels (ENaC)[5] located at the apical membranes, their extrusion across the basolateral membranes by the electrogenic Na^+-K^+-ATPase, and the passive movement of K^+ ions through basolateral K^+ channels. The entry of Na^+ ions through apical pathways is thought to be the rate-limiting step in this process (1–3). To preserve neutrality, Cl^- ions follow Na^+ ions both through transcellular and paracellular pathways (4, 5). The coordinated movement of Na^+ and Cl^- ions creates an oncotic gradient favoring the absorption of alveolar fluid.

Injury to either apical or basolateral pathways by partially reduced intermediates may lead to impairment of fluid reabsorption, which in turn may result in pulmonary edema, hypoxemia, and eventually death from respiratory failure (6–9). One such specie is hypochlorous acid (HOCl)[6], which may be generated either endogenously or exogenously. Millimolar concentrations of HOCl may be generated by activated neutrophils and eosinophils by the catalytic actions of neutrophil- and eosinophil-derived myeloperoxidases on chloride (Cl^-) and hydrogen peroxide (H_2O_2) in close proximity of the apical and basolateral membranes of epithelial cells (10, 11). The main targets of HOCl and its conjugated base (hypochloride:OCl^-) are free functional groups of proteins and amino acids, predominantly sulfhydryl groups (12, 13), free amine groups of plasma amino acids (yielding chlorinated amines; (14)), and aromatic amino acids (yielding chlorotyrosine (15–17)). Moreover, the reaction of HOCl with plasma or alveolar nitrite may form reactive intermediates capable of nitrating, chlorinating, and dimerizing aromatic amino acids, thus, damaging a number of key proteins and altering their functions (18).

In addition to endogenous sources, significant amounts of HOCl are also generated during chlorine (Cl_2) inhalation. We have shown that exposure of rats to Cl_2 causes extensive injury to the alveolar epithelium as manifested by changes in surfactant function and increased permeability to plasma proteins (19). Furthermore, exposure of surfactant protein A (SP-A), a critical component of innate immunity to HOCl, oxidized and

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[5] The abbreviations used are: ENaC, amiloride-sensitive epithelial sodium channel; hENaC, human ENaC; AFC, alveolar fluid clearance; HPLC, high performance liquid chromatography; AMC, 7-amido-4-methyl coumarin; Boc, *t*-butyl butoxycarbonyl; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; DTT, dithiothreitol.

[6] Throughout this work, we use the term hypochlorous acid (HOCl) to refer to the sum of hypochlorous acid and hypochlorite (OCl^-) that are present at equilibrium. The actual concentration of these species is governed by the pH and the pK_a of HOCl.

chlorinated amino acids in its carbohydrate recognition domain and decreased its ability to bind mannose residues, an important step in the binding and killing of pathogens (20). However, the effects of Cl_2 inhalation on Na^+-dependent alveolar fluid clearance (AFC) *in vivo* as well as the mechanisms by which Cl_2 and HOCl damage ENaC have not been investigated. This is an important area of research as damage to ENaC has been associated with abnormal fluid transport in various forms of lung injury (7, 21–25).

To address these questions we performed a number of physiological, biophysical, and biochemical studies. In our first series of experiments, we exposed mice to Cl_2 at concentrations likely to be encountered in industrial accidents or deliberate release of Cl_2 into the atmosphere (19, 26, 27) and measured Na^+-dependent AFC *in vivo* at various times post exposure after the mice were returned to room air. Because our results indicated that exposure to Cl_2 decreased AFC, we performed additional studies in *Xenopus* oocytes injected with cRNAs of the human ENaC (hENaC) subunits (α-, β-, and γ-ENaC) to identify the cellular and molecular mechanisms by which HOCl (formed by the hydrolysis of Cl_2 gas) as well as reactive intermediates formed by the reaction of HOCl with components of the media decreased Na^+- and amiloride-sensitive currents (I_{Na} and I_{amil}, respectively). Finally we found that when ENaC channels were locked in the open position either by the action of serine proteases (28–30) or the introduction of a point mutation in βENaC (31), HOCl did not decrease I_{Na}. We concluded that HOCl inhibits ENaC by altering channel gating and preventing closed channels from opening.

MATERIALS AND METHODS

Chemicals—NaOCl, *N*-acetylcysteine, amiloride, HEPES, bovine serum albumin, and dithiothreitol (DTT) were purchased from Sigma. Trypsin, elastase, and plasmin were purchased from Invitrogen, Worthington (Lakewood, NJ), and Molecular Innovations (Novi, MI), respectively.

Animals—6–8-Week-old BALB/c and C57BL/6 male mice (20–25 g body weight) were purchased from Charles River Laboratories (Wilmington, MA). They were housed in the animal unit for at least 3 days before any experimental procedure where they were provided with mouse chow and water *ad libitum*. All experimental protocols were approved by the Institutional Animal Care and Use Committees of the University of Alabama at Birmingham.

Exposure of Mice to Cl_2 Gas—Mice were exposed to Cl_2 gas (0–500 ppm) for 30 min as previously described (19). In brief, they were placed inside a cylindrical glass chamber (Specialty Glass, Inc. Houston, TX, #X02AI99C15A57H5). Two mass flow controllers with Kalrez seals (Scott Specialty Gases, Los Angeles, CA, #05236A1V5K) and a microprocessor control unit (Scott Specialty Gases, #05236E4) were used to control the flow rates of compressed air and Cl_2 (1000 ppm Cl_2 in air; Airgas, Birmingham, AL) to achieve the desired Cl_2 concentrations (0–500 ppm) in the exposure chamber. A bubble flow meter was used to determine the accuracy of each mass flow controller flow rate on a weekly basis. Air and Cl_2 were mixed at a three-way junction, and they were further mixed by passing through a diffuser located inside the top lid of the exposure chamber. Gases exited the chamber via two large-bore-diameter ports in its bottom half. The exposure chamber was placed inside a chemical hood located in a negative pressure room. At the end of the exposure period (30 min) the Cl_2 gas was turned off, the chamber was vented with compressed air for 2–3 min, the two halves were separated, and the mice were removed and returned to room air.

AFC Measurements—Alveolar fluid clearance was measured as previously described (7, 23). In brief, mice were anesthetized and paralyzed by intraperitoneal injections of diazepam (10 mg/kg body weight; Hospira, Lake Forest, IL), ketamine (200 mg/kg; IVX Animal Health, St. Joseph, MO), and pancuronium bromide (0.04 mg; Gensia Pharmaceuticals, Irvine, CA). They were ventilated with a mouse respirator (model 687; Harvard Apparatus, Holliston, MA) with 100% O_2, a tidal volume of 0.2 ml (9–10 ml/kg body weight), and frequency of 160 breathes/min through an 18-gauge intravenous catheter trimmed to about 0.5 inch, inserted into their tracheas. They were then placed in the left decubitus position on a heating pad (Braintree, Cambridge, MA); body temperature was maintained at 37–38 °C with a heating lamp. A 0.9% NaCl solution (0.3 ml; ~30% of their total lung capacity) containing 5% fatty acid-free bovine serum albumin (Sigma) was instilled via the tracheal catheter followed by 0.1 ml of room air to clear the dead space and position the fluid in the distal lung (alveolar) space. The osmolarity of the instillate, measured by a vapor pressure osmometer (Wescor, Inc; Logan, UT) was 322 mosmol/kg. In some cases amiloride (1.5 mM) was added in the instillate to inhibit ENaC. Previous measurements have shown that significantly higher concentrations of amiloride are needed to inhibit ENaC in air-filled lungs (7). After 15 or 30 min of ventilation, the instilled fluid was gently aspirated and kept in a 0.5-ml tube. Samples visually contaminated by blood were excluded. Protein concentrations were measured using the bicinchoninic acid protein assay (Pierce). A standard curve was prepared by assaying known concentrations of bovine serum albumin in 0.9% NaCl. AFC values were calculated as described before (24, 32). In some cases mice were anesthetized with isoflurane 15 min post-Cl_2 exposure, and 100 μl of saline containing either trypsin (5 μM) or vehicle were instilled dropwise in their nostrils. The isoflurane was discontinued, the mice woke up shortly after, and their AFC was measured 1 h post-exposure as described above.

HOCl Preparation—The concentration of HOCl was determined spectrophotometrically (Beckman DU-7400) from its absorbance at 292 nm (molar absorption coefficient (ϵ) = 350 M^{-1} cm^{-1}) at pH 12 (33). Stock solutions, prepared fresh every day, were then diluted in oocyte incubation medium (ND96, containing 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 10 mM HEPES at pH 7.6 (osmolarity, 200–220 mosmol)) to the desired final concentration just before each experiment.

Construction of ENaC cRNAs—Human α-, β-, and γ-ENaC cDNAs were subcloned into a pCDNA3.1 vector for *in vitro* transcription. hENaC-α595x was excised by NotI and Xho and cloned into pCDNA3.1 at NotI and XhoI. hENaC βS520K (provided by Dr. Peter Snyder, University of Iowa) were excised by NotI and Acc65I and cloned into pCDNA3 at NotI and XhoI,

with Acc65I and XhoI blunt-ended. hENaC-γ575x was excised by NotI and EcoRI and cloned into pCDNA3.1 at NotI and XhoI, with EcoRI and XhoI blunt-ended. All the constructs were verified by DNA sequencing. ENaC subunit plasmids were linearized, and cRNAs were prepared with a cRNA synthesis kit (T7 Message Machine, Ambion Inc, Austin, TX) according to the manufacturer's protocol. cRNAs were dissolved in RNase-free water, and their concentrations were determined spectrophotometrically. To facilitate the detection of exogenously expressed α-, β-, and γ-hENaC in Western blotting studies, in some cases α, β, and γ cDNAs were tagged with FLAG, Myc, and V5 epitopes at their C termini, respectively, and tagged-cRNAs were generated as above. All procedures have been described in detail previously (34).

Expression of ENaC in Xenopus Oocytes—Ovarian tissue containing oocytes from adult female *Xenopus laevis* toads was dissected under anesthesia, then digested in 2 mg/ml collagenase (type 1A, Roche Applied Science) in Ca^{2+}-free OR-2 medium, containing 82.5 mM NaCl, 2.0 mM KCl, 1.0 mM $MgCl_2$, and 5 mM HEPES, pH 7.4, under rotation at room temperature for 2 h as previously described (34). Defolliculated oocytes were washed 3 times in Ca^{2+}-free OR-2 medium followed by OR-2 medium with 1.0 mM $CaCl_2$. After that, stage VI oocytes were selected and cultured in half-strength Leibovitz-15 medium (Invitrogen) containing 15 mM HEPES, penicillin (100 units/ml), streptomycin (100 μg/ml), and 5% horse serum, pH 7.6, at 16 °C. In all cases equal amounts (8.3 ng each) of cRNAs were injected into oocytes in a total volume of 50 nl (0.5 μg/μl in RNase free water) per oocyte via a Nanoject microinjector (Drummond, Broomall, PA). Oocytes were then incubated in half-strength L-15 medium for 24 or 48 h at 16 °C until use. The culture medium was changed every other day.

Voltage Clamp Recordings of Whole-cell ENaC Currents—Whole-cell cation currents were recorded 48–72 h post-injection across the entire oocyte membrane using the two-electrode voltage clamp technique (35). Briefly, the oocytes were held in a small groove in a chamber of 1-ml volume at room temperature (21 °C). The chamber was filled with ND96 solution. The oocytes were impaled with two 3 M KCl-filled electrodes with resistances of 0.4–2.2 megaohms. The recording electrodes were constructed from glass micropipettes (Rochester Scientific, Rochester, NY) with a two-stage vertical Narishige PC-10 microelectrode puller (Narishige Scientific Instrument Laboratory, Tokyo, Japan). While in the chamber, oocytes were perfused with ND96 at a flow rate of 3 ml/min using a Warner Six Channel Valve Control Systems apparatus (Warner Instruments, Hamden, CT). Oocytes voltage clamping and currents recordings were accomplished with a Dagan TEV-200A voltage clamp amplifier (Dagan Corp., Minneapolis, MN). Two reference electrodes were connected to the bath. Oocytes were clamped at a holding potential of −40 mV. Currents were continuously monitored and recorded on a chart recorder and to the hard disk of a personal computer. The currents were elicited by varying oocyte membrane potentials from −140 to +60 mV every 10 s after a standard protocol (starting from holding potential for 50 ms, then to −140 mV for 400 ms, subsequently back to −40 mV for 200 ms, and to +60 mV for 400 ms, finally back to −40 mV for 50 ms) generated by pCLAMP 8.0 software (Axon Instruments, Union City, CA). Current-voltage (I-V) relationships were acquired while the membrane potentials were changed from −120 to + 80 mV in 20-mV increments ever 500 ms. In some cases, after obtaining base-line I-V relationships and recording stable currents at −140 mV under control conditions, oocytes were perfused with ND96 mixed with various concentrations of HOCl for 10 min at which time recordings of the I-V relationship were repeated. Amiloride (10 μM) was then added in the bath solution, and amiloride-sensitive currents (I_{amil}) were calculated as the difference currents before and after perfusion with amiloride. In other cases, oocytes were incubated with ND96 with various concentrations of HOCl for 2–4 h before the recordings of I_{amil}.

Single-channel ENaC Current Recordings—Oocytes were incubated for 10 min with various concentrations of HOCl in ND96. They were then washed in ND96, and their vitelline membranes were removed mechanically by immerging them into a hypertonic solution containing 200 mM sucrose. Patch-clamp pipettes were prepared from borosilicate glass capillaries (Sutter Instruments Co., Novato, CA) using a two-stage vertical Narishige PC-10 microelectrode puller (Narishige Scientific Instrument Laboratory, Tokyo, Japan) and then fire-polished using a microforge (MF-830, Narishige, Japan). The pipette resistance when filled with ND96 was 7–10 megaohms. Currents were recorded at +100 mV depolarizing potential applied to the on-cell patch at a sampling rate of 5 kHz and filtered at 1 kHz with a low-pass Bessel filter through an Axopatch 200B amplifier (Axon Instruments). Data were analyzed using pCLAMP 9 software. Only data from patches with a seal resistance of at least 10 gigaohms and stable currents for at least 5 min of recordings were included in the final analysis. All experiments were carried out at room temperature.

Liquid Chromatography-Mass Spectrometry Analysis—Reactive intermediates formed by the interaction of HOCl with components of the ND96 medium were detected by liquid chromatography-mass spectrometry analysis. Briefly, samples were diluted 1:10 in water, then separated with an HPLC system (Shimadzu Class VP, Kyoto, Japan) at 4 °C consisting of an LC-10AD pump and a SIL-HTC Autosampler, with an injection volume of 20 μl and flow rate of 0.2 ml/min through a C5 column (250 × 2.0 mm, Phenomenex; Torrance, CA). The two mobile phase solvents (A and B) consisted of 0.1% HCOOH (A) and methanol and 0.1% formic acid (B). The column was first equilibrated in solvent A. After sample injection, solvent B was increased from 0 to 80% within 10 min, then switched back to 0% until the flow was stopped at 17 min. The eluted material was passed into the electrospray ionization interface of a MDS/Sciex Applied Biosystems API 3200 (Foster City, CA) operating in the positive ion mode. Mass spectra were recorded over a 70–500 *m*/*z* range.

Determination of Membrane Protease Enzymatic Activity—Serine protease activity in the oocytes membranes was monitored by measuring the release of a fluorescent probe (7-amido-4-methyl coumarin (AMC)) from a peptide substrate *t*-butoxycarbonyl (Boc)-Gln-Ala-Arg-AMC-HCl; R&D Systems, Minneapolis, MN) using a fluorescence plate reader (FLUOstar OPTIMA, BMG Labtech; Durham, NC) (34). Briefly, five oocytes each, incubated with either HOCl (2 mM) or

vehicle in ND96 for 10 min, were added to a cuvette containing 200 μl of ND96 medium, and the reaction was started by adding Boc-Gln-Ala-Arg-AMC-HCl (50 μM final concentration). The reaction was monitored for up to 20 min by recording fluorescence at 460 nm after excitation at 380 nm.

Detection of ENaC Protein in Oocyte Plasma Membranes—*Xenopus* oocyte plasma membranes were purified as previously described (36) with minor modifications. Briefly, oocytes were washed twice in Barth solution (90 mM NaCl, 3 mM KCl, 0.82 mM $MgSO_4$, and 5 mM HEPES, pH 7.6) and homogenized in 0.8 ml of the same solution supplemented with protease inhibitor mixture (Sigma). Homogenates were centrifuged at 250 × *g* for 10 min at 4 °C, and the supernatant was centrifuged at 16,000 × *g* for 20 min at 4 °C to pellet down total membranes. Pellets were lysed in 1% Triton-100 lysis buffer (150 mM NaCl, 50 mM Tris-Cl, pH 7.4, 2 mM EDTA, 1% Triton-X100, protease inhibitor mixture), and the lysates were centrifuged at 16,000 × *g* for 20 min at 4 °C. The supernatants consisted of plasma membrane extracts. Equal amounts of whole oocyte or plasma membrane proteins were separated by 8% polyacrylamide-SDS gels. After transfer onto polyvinylidene difluoride membrane, the blots were probed for α-, β-, and γ-hENaC using antibodies to FLAG, Myc, and V5 epitopes (Sigma) respectively. The level of glyceraldehyde-3-phosphate dehydrogenase was also determined to demonstrate equal loading of the extracts and the purity of the plasma membrane extracts.

Construction and Application of a Protease Target Peptide in γENaC—A 51-residue peptide between the putative furin and plasmin cleavage sites on the γ-hENaC (N-EAESWNSVSEGK-QPRFSHRIPLLIFDQDEKGKARDFFTGRKRKVGGSIIHK-C) was synthesized by the solid phase method using a Protein Technologies Model PS3 Automated Peptide Synthesizer (Rainin Instrument Co., Inc; Woburn, MA) by the UAB Peptide Synthesis Core facility. The first amino acid from the C terminus Fmoc-Lys-(Boc)-OH was derivatized to the solid support using the Wang Resin (100–200 mesh substitution 0.83 mmol/g, Nova Biochem, Damstadt, Germany). The Fmoc group was then removed using piperidine in dimethylformamide. The rest of the amino acids in the sequence were coupled to the first amino acid in the presence of 2-(H-benzotriazole-1-yl)-1,1,3,3,-tetramethlyuronium hexafluorophosphate (HBTU) and *N*-methylmorpholine. After the addition of the final amino acid, the amino protecting Fmoc group was cleaved, and the peptide was released from the resin, with the removal of the side-chain protections used for the various amino acids in the sequence using trifluoroacetic acid in the presence of scavengers. The crude peptide was purified using reverse phase HPLC. The purity of peptide was determined by HPLC followed by mass spectral analysis.

Xenopus oocytes expressing hENaC were perfused for 15 min with either ND96 containing the native peptide (3 μg/ml) or ND96 containing peptide (3 μg/ml) mixed with 2 mM HOCl for 10 min. Whole cell inward Na^+ currents at −100 mV were continuously recorded, and amiloride-sensitive current voltage relationships were obtained at the end of 10-min recordings.

Data Analysis—All data were shown as the means ± S.E. Comparisons between data sets were made with unpaired or paired *t* test. Multiple comparisons among treatment groups were performed using analysis of variance. Differences at $p <$ 0.05 were considered significant.

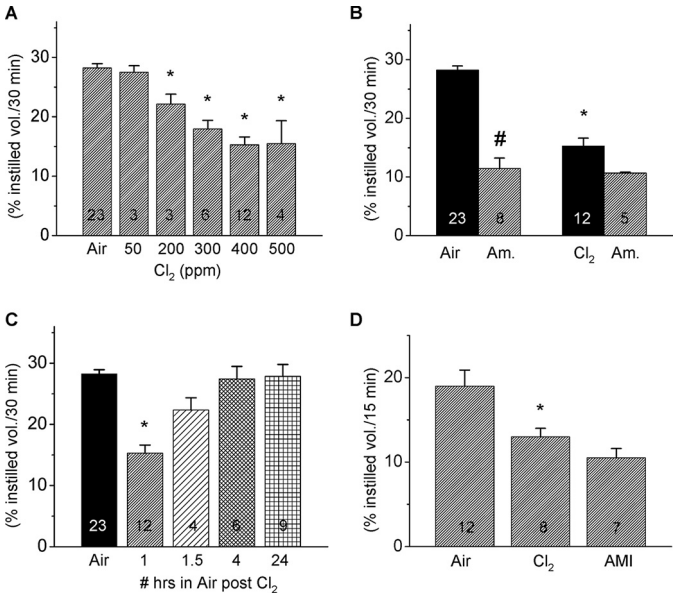

FIGURE 1. **Exposure of mice to Cl_2 decreases alveolar fluid clearance.** *A*, C57BL/6 mice were exposed to the indicated concentrations of Cl_2 gas for 30 min. Alveolar fluid clearance (expressed as % of instilled volume per 30 min) was measured 1 h post-exposure in anesthetized and ventilated mice as described under "Materials and Methods." *B*, C57BL/6 mice exposed to 400 ppm Cl_2 for 30 min. Alveolar fluid clearance was measured 1 h post-exposure. Amiloride (*Am.*; 1.5 mM final concentration) or an equivalent volume of vehicle was added in the instilled solution in some animals. *C*, measurements of AFC in BALB/C mice exposed to 400 ppm Cl_2 and returned to room air for the indicated intervals. *D*, BALB/c mice were exposed to 400 ppm Cl_2 for 30 min. Alveolar fluid clearance was measured in the presence and absence of amiloride (*AMI*; mean ± S.E.; *, $p < 0.05$ compared with the corresponding air value; #, $p < 0.05$ compared with the corresponding value without amiloride).

RESULTS

Exposure of Mice to Cl_2 Decreases Their AFC—Previous studies documented the presence of large variations in physiological responses of different species of mice to oxidative stresses (37). For this reason, we exposed both C57BL/6 and BALB/c mice to various concentrations of Cl_2 (50–500 ppm for 30 min) and measured AFC at various times post-exposure. As shown in Fig. 1*A*, exposure of C57BL/6 mice to increasing concentrations of Cl_2 gas (50–500 ppm for 30 min) decreased AFC from 28 ± 0.7 to 15 ± 4% (% of instilled volume/30 min, $n = 3$–23, $p < 0.05$) at 1 h post-exposure. Data shown in Fig. 1*B* (400 ppm, $n = 4$–23) indicate that Cl_2 or its reactive intermediates decreased the amiloride-sensitive fraction of AFC, consistent with specific damage to ENaC. Furthermore, as shown in Fig. 1*C*, the AFC values of mice exposed to Cl_2 and returned to room air increased toward their base-line values within 90 min post-exposure, a time course consistent with the half-life of ENaC in epithelial cells ($t_{1/2} = 1$ h) (38). Similar results were obtained in BALB/c mice (Fig. 1*D*). These data indicate that the observed decrease of AFC was not due to nonspecific injury to the alveolar epithelium.

Inhibition of I_{Na} in hENaC-expressing Oocytes by HOCl—To more definitively demonstrate that HOCl damaged ENaC, we recorded whole cell and single channel Na^+ currents across

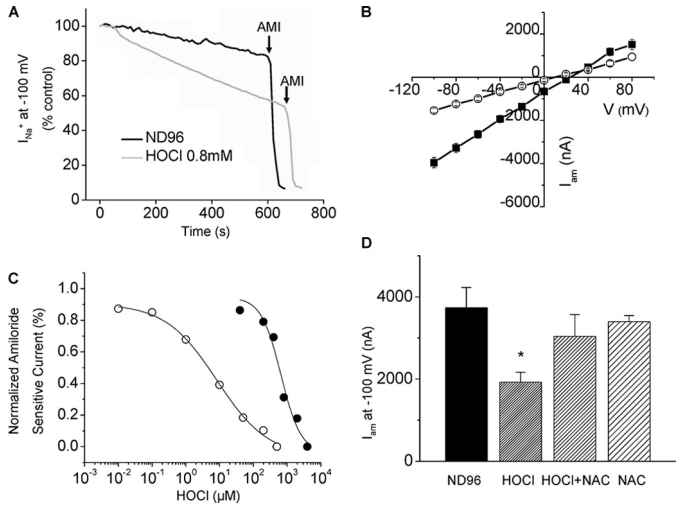

FIGURE 2. **HOCl decreases Na^+ currents across *Xenopus* oocytes injected with α-, β-, and γ-hENaC.** *A*, oocytes injected with hENaC were perfused with either ND96 or a solution in which 0.8 mM HOCl was added in ND96 (0.8 mM HOCl-ND96). As mentioned under "Results" and shown in Fig. 4, the fast reaction of HOCl with HEPES and other compounds in ND96 mainly generated organic chloramines. Inward ENaC currents were measured by pulsing the membrane potential from its resting value (−40 mV) to −140 mV for 500 ms. Amiloride (*AMI*, 10 μM) was added into the perfusion medium at the times indicated by the *arrows*. To better demonstrate the effects of HOCl, the results were expressed as % of their corresponding base-line values before the addition of HOCl. Shown are characteristic tracings that were repeated using 10 different oocytes from three different isolations. *B*, current-voltage relationships of amiloride-sensitive difference (I_{am}) currents were obtained after perfusion of hENaC-expressing oocytes with either 0.8 mM HOCl-ND96 (*open circles*) or ND96 alone (*closed squares*) for 10 min. *C*, normalized amiloride-sensitive currents across hENaC-expressing oocytes were either incubated (*open circles*; $n \geq 8$ for each data point) or perfused (*solid circles*; $n \geq 10$ for each data point) with the indicated concentrations of HOCl-ND96 for 2 h and 10 min, respectively. Normalized amiloride-sensitive currents were calculated as follows as previously described (77); $I = 1 - (I_o - I_x)/(I_o - I_{max})$, where I_o and I_x are the I values in the absence of HOCl and the maximum HOCl added. Inhibition constants (k_i) were calculated using the Origin software by fitting the data points with the following equations; $I = (1 - [1/(1 + K_i/x)])I_{max}$, where I_{max} is the maximum current (*i.e.* the current in the absence of HOCl), and x is the concentration of HOCl. For clarity, only mean values (without S.E.) are shown. *D*, amiloride-sensitive currents (I_{am}) at −100 mV for hENaC-expressing oocytes were perfused with HOCl (400 μM), a mixture of HOCl (400 μM), and *N*-acetylcysteine (*NAC*, 1 mM) or *N*-acetylcysteine (1 mM) alone (mean ± S.E.; $n \geq 10$ oocytes for each group. *, $p < 0.05$ compared with ND96 alone).

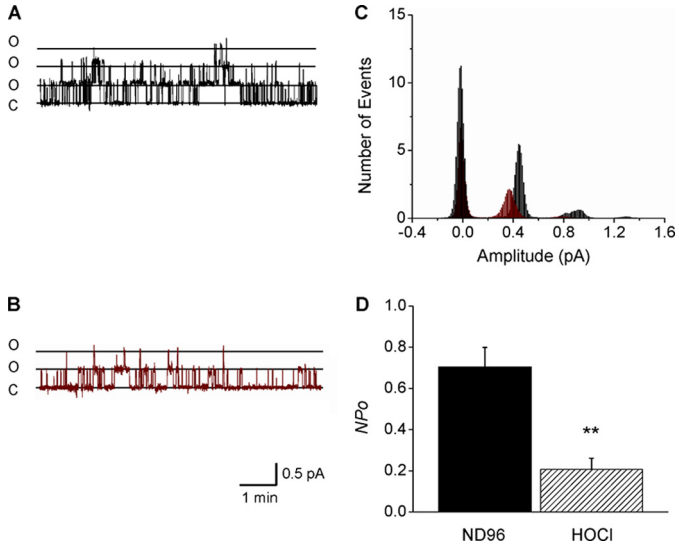

FIGURE 3. **HOCl decreases Na^+ single channel activity in cell-attached patches of hENaC-expressing oocytes.** Oocytes were patched in the cell-attached mode as described under "Materials and Methods," currents were measured at a pipette holding potential of −100 mV ($V_{holding} = V_{apical} - P_{pipette}$), and amplitude distribution histograms were generated as described under "Materials and Methods." Control (*A*) and 10-min post-perfusion with 2 mM HOCl-ND96 record (*B*) is shown. Open (*O*) and closed (*C*) states are indicated on the records. Notice a visible decrease of open state of single channels in HOCl-perfused oocytes. Typical records are from five control and five HOCl-perfused oocytes from two different batches. *C*, amplitude histograms for single channels from control (*black*) and HOCl perfused (*red*) oocytes are shown in *A* and *B*. *D*, NP_o values were calculated from all amplitudes histograms as mentioned under "Materials and Methods" (mean ± S.E.; $n = 5$; **, $p < 0.01$).

hENaC-expressing oocytes either during continuous perfusion or incubation with various concentrations of HOCl. For technical reasons, we were unable to expose oocytes to Cl_2 gas. As discussed below, our inability to detect HOCl in ND96 (the oocyte recording medium) at the time of perfusion led us to conclude that HOCl reacted with some of the ND96 components to form intermediates, such as organic chloramines, which may have been responsible for the observed effects (39). Thus, in this system, by HOCl-induced injury, we refer to HOCl *per se* and all formed reactive intermediates.

As shown in the various panels of Fig. 2, *Xenopus* oocytes injected with α-, β-, and γ-hENaC expressed significant amounts of inwardly rectified I_{Na}, more than 90% of which were rapidly inhibited by the addition of amiloride (10 μM) into the bath. Perfusion of hENaC-expressing oocytes with ND96 containing HOCl (0.8 mM) for 10 min significantly inhibited both the basal (Fig. 2*A*) and the amiloride-sensitive currents (Fig. 2*B*) with a half-maximum effect at about 600 μM (Fig. 2*C*). When hENaC-expressing oocytes were incubated with ND96 containing HOCl for 2 h, which better mimics the *in vivo* situation where activated neutrophils may generate significant amounts of HOCl in close proximity of ENaC over long periods of time, significant inhibition of I_{amil} was noted at a much lower concentration (half-maximum concentration = 4 μM; Fig. 2*C*). Subsequent addition of a variety of reducing agents in the medium (10 mM DTT or 1 mM *N*-acetylcysteine) did not reverse I_{amil} by a significant amount (data not shown), indicating that ENaC inhibition was due to the formation of irreversible oxidation states in key amino acids. On the other hand, perfusion of oocytes with a mixture of ND96 containing HOCl (400 μM) and *N*-acetylcysteine (1 mM), which scavenged HOCl to non-detectable levels, totally prevented the observed decrease of I_{amil} (Fig. 2*D*). The addition of HOCl did not significantly alter the pH or the Cl^- concentration of the medium, two variables shown to modulate ENaC activity in previous studies (40, 41).

HOCl Inhibits Single-channel Na^+ Currents in hENaC-expressing Oocytes—In our next series of studies, we recorded single-channel currents from hENaC-expressing oocytes using the cell-attached configuration. Forty eight hours post-injection with hENaC, oocytes expressed Na^+ single channels with unitary conductances of about 4 picosiemens (Fig. 3, *A* and *C*) with long open and closed times, characteristic of ENaC. Perfusion of oocytes with 2 mM HOCl in ND96 for 10 min decreased both the number (*N*) and the open probability (P_o) of ENaC (Fig. 3, *B* and *C*). ENaC conductance was not altered. An analysis of the representative control current recording in Fig. 3*A* showed the following values for the open probabilities (P_o) of various open levels: level 1, $P_o1 = 0.262$; level 2, $P_o2 = 0.13$;

level 3, $P_o3 = 0.016$; overall $NP_o = 0.57$. The values for the representative HOCl-treated oocyte recording shown in Fig. 3*B* were: $P_o1 = 0.21$; $P_o2 = 0.017$; overall $NP_o = 0.25$. Single channel NP_o (calculated from all point histograms from recordings of at least 10 min) were 0.70 ± 0.10 for control oocytes and 0.21 ± 0.05 after perfusion with 2 mM HOCl for 10 min (Fig. 3*D*; means ± S.E.; $n = 5$; $p < 0.01$).

Detection of Organic Chloramines—As shown in Fig. 4*A*, mass spectrometry analysis of products formed 1 h after the addition of HOCl (2.5 mM) into ND96 revealed the presence of various chloramine-type compounds. These agents were still present (albeit in smaller concentrations) 6 h later (Fig. 4*B*). On the other hand, chloramines were not detected when HOCl was added into ND96 containing *N*-acetylcysteine (Fig. 4*C*) or when HOCl was added in phosphate buffer that did not contain HEPES (data not shown). We concluded that these compounds were most likely formed by the interaction of HOCl with HEPES, which is included traditionally in ND96 as a buffer. This is in agreement with previous reports suggesting that HOCl interacts with HEPES to form chloramine-like compounds (39, 42).

Chloramines Formed by HOCl Inhibit I_{Na}—We performed a series of studies to test the hypothesis that stable reactive intermediates formed by the interaction of HOCl with ND96 were mainly responsible for the inhibition of ENaC activity *in vitro*. We synthesized chloramines by mixing excess amounts of either glycine or taurine (10 mM) with HOCl (2 mM) in phosphate buffer that lacked HEPES. We chose taurine and glycine because these amino acids exist in high concentrations in human plasma (162 ± 60 and 236 ± 42 μM, (43)), and they react with high rate constants with hypochlorite ($4.8 \pm 0.1 \times 10^5$ and $0.65–1.5 \times 10^5$ M^{-1} s^{-1}, respectively (14, 44)), suggesting their corresponding chloramines may be formed *in vivo*. As shown in Fig. 5*A*, perfusion of hENaC-expressing oocytes with the mixture of glycine and HOCl significantly inhibited I_{Na} as compare with glycine alone ($p < 0.05$, $n = 6$). The same results were obtained with the taurine and HOCl mixture (Fig. 5*B*). Subsequent addition of DTT (10 mM) had no effect on I_{Na}, indicating the presence of irreversible oxidation states.

Serine Proteases Increased Na^+ Currents after Inhibition by HOCl—Serine proteases, such as trypsin, plasmin, and elastase, have been reported to enhance ENaC activity by proteolytic cleavage of the α- and γ-ENaC subunits (45–49). We investigate whether extracellular serine proteases reversed the inhibitory effects of HOCl and its reactive products formed by the interactions with components of ND96. We perfused hENaC-expressing oocytes with ND96 containing HOCl for 10 min, which decreased I_{Na} in a time-dependent fashion (Fig. 6*A*). At the end of this period we perfused oocytes with ND96 containing trypsin (100 nM), elastase, or plasmin (10 μg/ml each). As shown in Fig. 6, *A–D*, perfusion of oocytes with proteases post-HOCl rapidly increased I_{Na} from about 20% of their control values to nearly normal levels rapidly. In contrast, perfusion with ND96 alone or ND96 containing DTT (up to 10 mM) had no effect on I_{Na} (data not shown). Subsequent addition of amiloride (10 μM) in the perfusion medium decreased the current by almost 90%, indicating that these proteases activated ENaC.

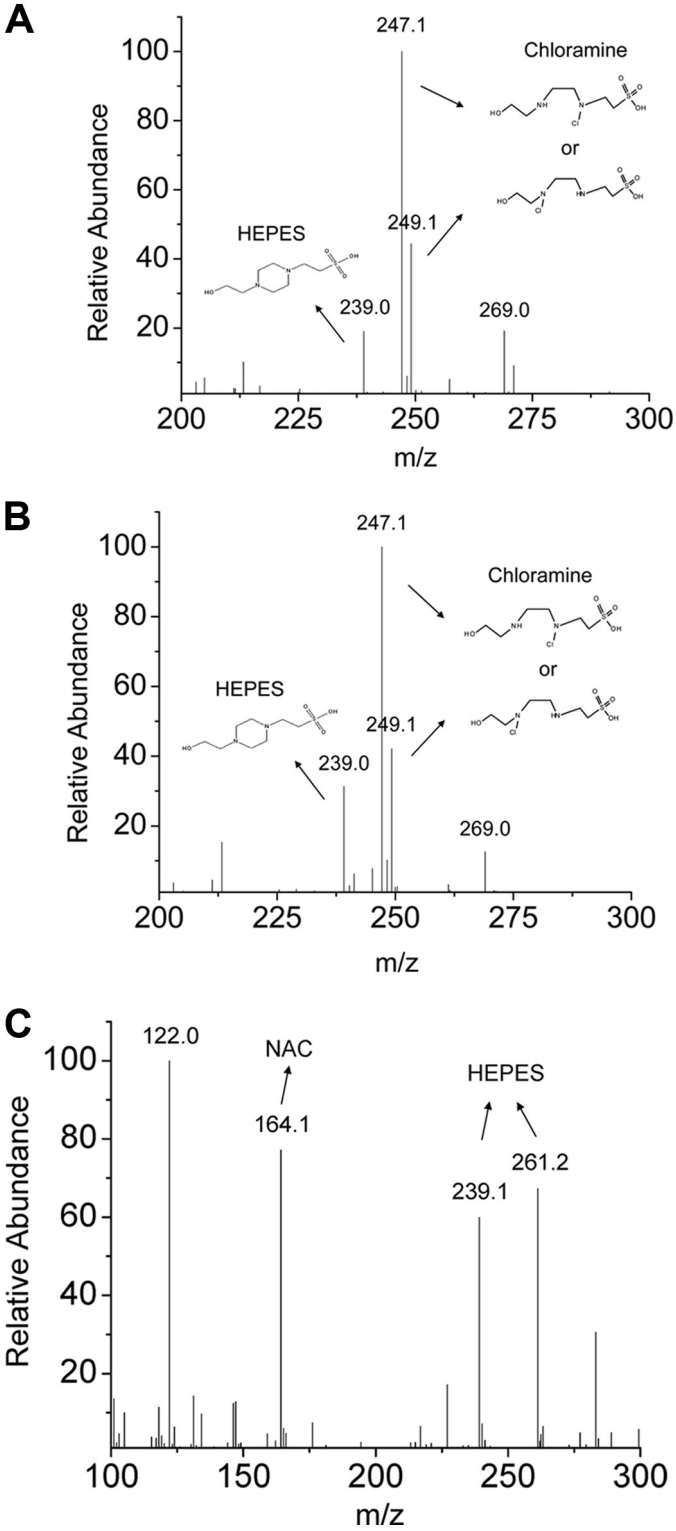

FIGURE 4. **Mass spectrometry analysis of reaction products formed by the reaction of HOCl with ND96.** 2.5 mM HOCl were added into ND96 for 1 and 6 h. Samples of medium were then analyzed with tandem mass spectroscopy as discussed under "Materials and Methods." Records show the mass-to-charge ratios (*m/z*) for the various fragments formed by the interaction of HOCl with ND96. Possible structures of the most abundant compounds (organic chloramines) formed after 1 (*A*) and 6 h (*B*) post-addition are shown as *insets*. Notice the absence of these organic chloramines 1 h post-HOCl (400 μm) addition into ND96 containing *N*-acetylcysteine (*NAC*, 1 mM) (*C*). Typical records were reproduced at least three times with identical results.

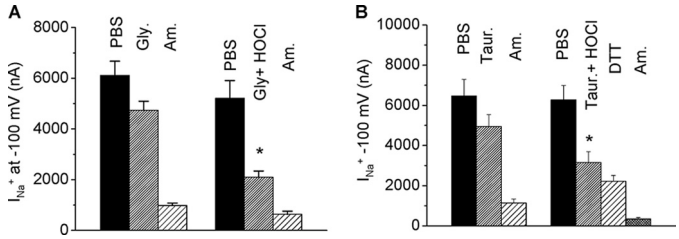

FIGURE 5. **Inhibition of I_{Na} by glycine and taurine chloramines formed in the absence of HEPES.** Inward Na^+ currents at −100 mV across hENaC-expressing oocytes perfused for 10 min with (*A*) glycine (10 mM) in phosphate buffer or glycine (10 mM) and 2 mM HOCl in phosphate buffer (*PBS*) and taurine (10 mM) in phosphate buffer or taurine (10 mM) and 2 M HOCl in phosphate buffer (*B*). Glycine or taurine were mixed with HOCl in phosphate buffer for 1 h before perfusion (no HEPES present). Amiloride (*Am.*, 10 μM) and DTT (10 mM) were added into the medium 10 min post perfusion (mean ± S.E. for the indicated number of oocytes; *, $p < 0.05$).

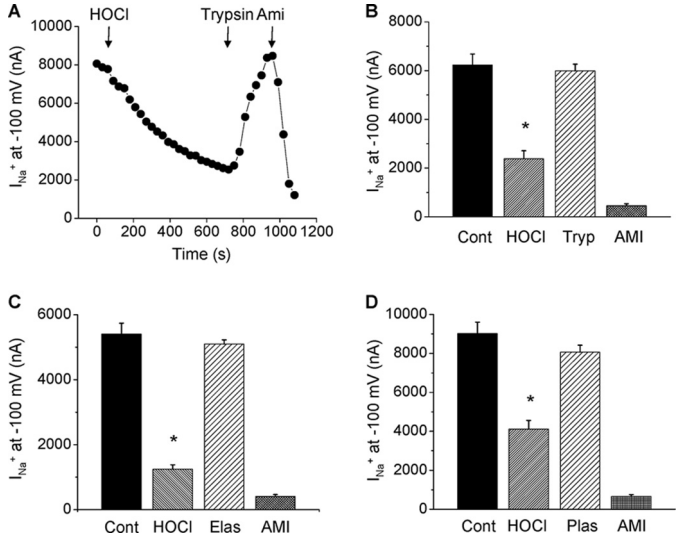

FIGURE 6. **Trypsin, plasmin, and elastase increase I_{Na} after HOCl inhibition.** *A*, hENaC-expressing oocytes were perfused with 2 mM HOCl-ND96 for 10 min. Inward Na^+ currents were measured continuously at −100 mV as described under "Materials and Methods." After 10 min, oocytes were perfused with ND96 containing 100 nM trypsin, which rapidly increased the currents to the control level. Subsequent addition of amiloride (*Ami*, 10 μM) into the perfusate decreased the currents to almost zero (recording was discontinued). *B–D*, mean values for the experiment are shown after perfusion with trypsin (*Tryp*, 100 nM; *B*; $n = 8$), elastase (*Elas*, 10 μg/ml; *C*; $n = 6$), or plasmin (*Plas*, 10 μg/ml; *D*; $n = 6$) (mean ± S.E., *, $p < 0.05$). *Cont*, control.

In the second set of this series of experiments, we first perfused hENaC-expressing oocytes with trypsin, elastase, or plasmin and then with HOCl (2 mM). As shown in Fig. 7, *A–D*, perfusion of oocytes with serine proteases for 5 min dramatically increased I_{Na} by 100%; subsequent perfusion with HOCl had no effect on I_{Na} (in contrast to more than 60% decrease in the absence of protease pretreatment). As shown in Fig. 6, the addition of proteases to ENaC-expressing oocytes post-HOCl exposure did not increase I_{Na} to the same extent as in control non-exposed oocytes (Figs. 6 and 7), most likely because proteases activate ENaC that have not been altered by HOCl. To test this hypothesis, we incubated ENaC-expressing oocytes with HOCl (100 μM) for 2 h, which decreased I_{Na} to almost zero, suggesting that under these conditions all membrane-bound ENaC were completely inhibited by HOCl (as shown in Fig. 2*C*). Under these conditions, subsequent perfusion of these oocytes with trypsin increased I_{Na} by less than 10% (Fig. 8, *A* and *B*). These data indicated that proteases added after HOCl treatment most likely acted on the subset of ENaC molecules that had not been affected by HOCl.

One possible explanation for the lack of effect of HOCl on I_{Na} after protease treatment is that proteases increased the number of mature ENaC channels, thus, decreasing the number of target ENaC molecules for HOCl. To investigate this possibility, we treated hENaC-expressing oocytes with various concentrations of trypsin, which resulted in a dose-dependent increase of I_{Na}, and then perfused them with HOCl in ND96 for 10 min. As shown in Fig. 9, HOCl had no effect on I_{Na} after trypsin treatment irrespective of the starting value of I_{Na}.

Membrane ENaC and Protease Activity Levels Were Not Decreased by HOCl—We measured membrane ENaC levels after perfusion of oocytes with HOCl (2 mM) for 10 min. As shown in Fig. 10, no significant changes in α-, β-, or γ-ENaC protein levels were seen. Furthermore, incubation of control and HOCl-treated oocytes with the fluorogenic peptide Boc-Gln-Ala-Arg-AMC-HCl (which were cleaved by membrane-bound proteases, thus, increasing fluorescence intensity in the supernatant) resulted in similar levels of fluorescence in the supernatant, indicating that HOCl and its reactive intermediates did not inactivate endogenous membrane proteases responsible for ENaC activation (Fig. 11).

Protease-cleaved ENaC Is Insensitive to HOCl Due to Changes in Its Gating—Several proteases, including furin, prostasin, plasmin, and elastase activate ENaC by cleaving segments in the finger domain within the large extracellular loop of its γ-subunit, which locks ENaC in the open state (Fig. 12*A*). Because our data showed that HOCl and its reactive intermediates had no effect on I_{Na} after protease activation, we hypothesized that functional injury to ENaC by HOCl required ENaC to be in its closed state. To test this hypothesis, we synthesized a 51-mer peptide mimicking the one released when plasmin cleaves γ-ENaC after it has been first cleaved by furin. As shown in Fig. 12*B*, I_{Na} values were increased 4 min after perfusion of hENaC-expressing oocytes with ND96-containing plasmin (10 μg/ml); however, subsequent perfusion of these oocytes with ND96 containing the 51-mer peptide (3 μg/ml) returned I_{Na} to their control levels. In a second set of experiments, oocytes were first perfused with plasmin (which increased I_{Na}) and then with ND96 containing both HOCl (2 mM) and the peptide (3 μg/ml). In this case I_{Na} was significantly inhibited by HOCl (Fig. 12*B*).

To further investigate the importance of proper ENaC gating in its inhibition by HOCl, we injected oocytes with wild type α- and γ-hENaC and a mutant form of βENaC (βS520K), which results in higher amiloride-sensitive currents by locking ENaC in their open state (Fig. 13*A*), in agreement with previous reports (31). The addition of trypsin had no effect on I_{Na} of these mutant channels; perfusion with 1 mM HOCl for 10 min or incubation with 100 μM HOCl for 2 h (which reduced I_{Na} of wild type ENaC to zero) had little effect on I_{Na} of these channels either (Fig. 13, *A* and *B*). The lack of effect of HOCl on these locked-open channels suggested HOCl inhibited ENaC by affecting channel gating. Sheng *et al.* (50) also reported that furin cleavage of ENaC relieves its self-inhibition by external $[Na^+]$, a mechanism putatively involving channel gating.

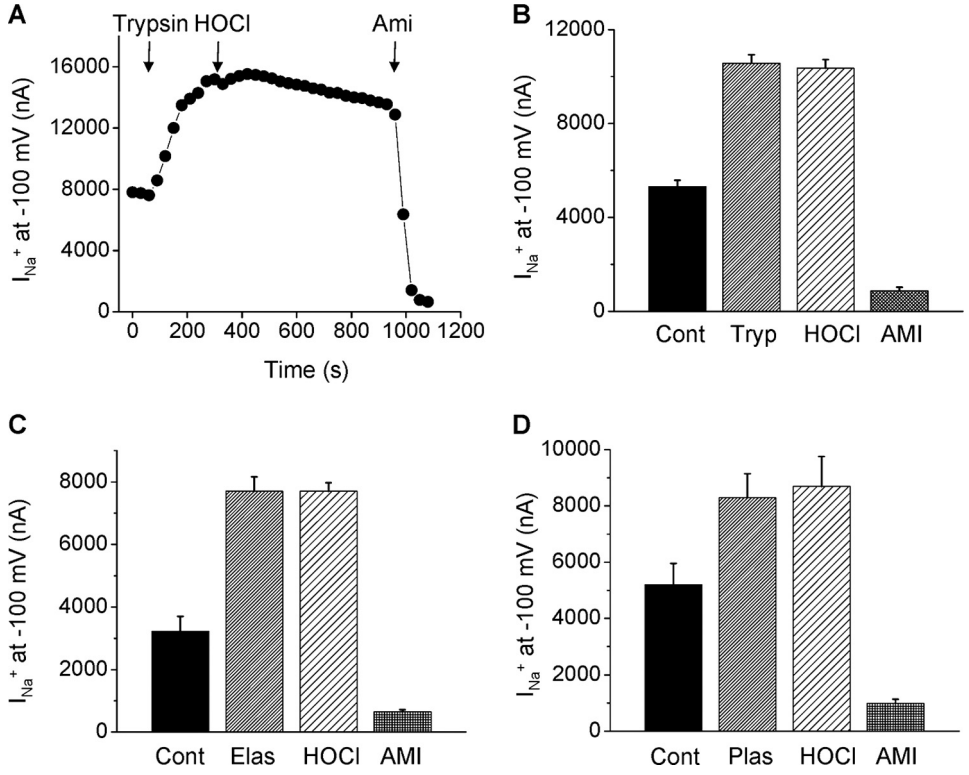

FIGURE 7. **Serine proteases renders ENaC insensitive to HOCl.** *A*, hENaC-expressing oocytes were perfused with ND96 containing 100 nM trypsin. Inward Na^+ currents were measured continuously at −100 mV as described under "Materials and Methods." After 5 min, when the I_{Na} had reached a new plateau, oocytes were perfused with 2 mM HOCl-ND96, which had no effect on I_{Na}. Subsequent addition of amiloride (*Ami*, 10 μM) into the perfusate decreased I_{Na} to almost zero. *B–D*, mean values are shown for the corresponding experiments after perfusion of hENaC-expressing oocytes with trypsin (*Tryp*, 100 nM; *B*; $n = 11$), elastase (*Elas*, 10 μg/ml; *C*; $n = 8$), or plasmin (*Plas*, 10 μg/ml; *D*; $n = 6$) (means ± S.E. *. $p < 0.05$). *Cont*, control.

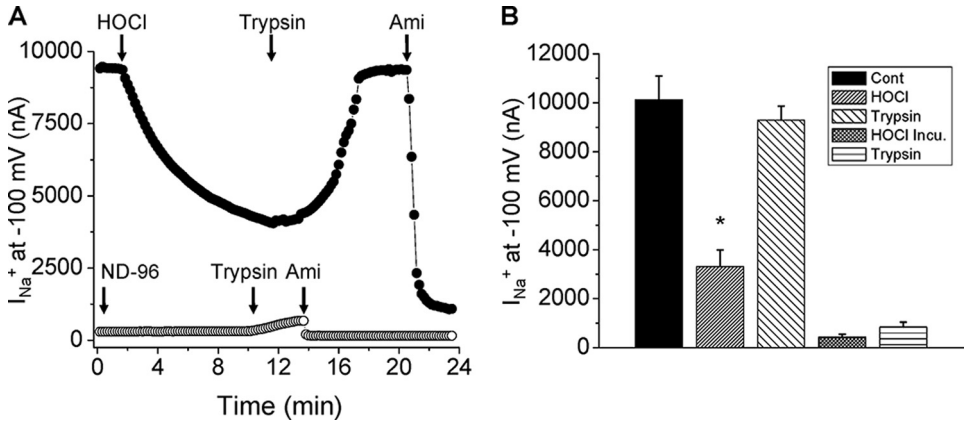

FIGURE 8. **Trypsin has little effect on I_{Na} inhibited by HOCl incubation.** *A*, shown is a representative time course recordings of I_{Na} at −100 mV from hENaC-expressing oocytes perfused by 1 mM HOCl-ND96 (●) or incubated in 100 μM HOCl-ND96 for 2 h (○), then perfused with trypsin (*Try*, 2 μM) and amiloride (*Ami*, 10 μM). *B*, group data showed trypsin increased I_{Na} to control levels in oocytes perfused with HOCl-ND96. Incubation (*Incu*) with HOCl-ND96 for 2 h greatly inhibited I_{Na}, and perfusion with trypsin had little effect on I_{Na} of these oocytes (mean ± S.E., $n = 11$ for HOCl-perfused and 5 for HOCl-incubated oocytes; *, $p < 0.05$).

was instilled dropwise in the nostrils. Mice recovered quickly, and 1 h post-exposure, they were anesthetized and ventilated, and AFC was measured. Exposure to Cl_2 decreased AFC by about 30% in agreement with the data shown in Fig. 1. However, normal values of AFC were seen in mice exposed to Cl_2 and instilled with trypsin (Fig. 14). Trypsin had no effects on AFC of control mice (in contrast to what was measured in oocytes), most likely because ENaC was maximally stimulated under our experimental conditions.

DISCUSSION

Hypochlorous acid/hypochlorite, as the major hydrolysis products of chlorine gas, and predominant oxidant produced by stimulated neutrophils play an important role in inducing lung edema as well as increasing pulmonary vascular permeability and arterial pressure (51). However, until recently, little was known about the effects of these agents on membrane ion channels, especially ENaC. Herein we demonstrate that reactive intermediates (such as organic chloramines and other reactive intermediates), formed by the action of Cl_2 and HOCl in concentrations likely to be encountered *in vivo* during pathological conditions (51–53) via interaction with components of the media and lung lining fluid, inhibit ENaC and decrease Na^+-dependent fluid clearance across the distal lung epithelium. Furthermore, the fact that serine protease both prevents and reverses the effects of these compounds on ENaC and the fact that HOCl has no effect on ENaC channels that remain constitutively open indicate that HOCl mainly prevents ENaC channels in the close state from opening, most likely via post-translational modifications of key amino acids. To the best of our knowledge this is the first report of this kind.

Intranasal Administration of Trypsin Post-Cl_2 Exposure Increases AFC—In the final set of experiments we assessed whether intranasal instillation of trypsin could reverse the decrease of AFC in mice exposed to Cl_2. Mice were exposed to chlorine (300 ppm for 30 min) and then returned to room air. Fifteen minutes post-exposure, they were briefly anesthetized with isoflurane, and trypsin (5 μM; dissolved in 100 μl of saline)

Cl_2 gas is not very soluble in water (Henry's law constant (K_H) = 6.2–9.5 × 10^{-2} M/atm or 6.2–9.5 × 10^{-8} M/ppm Cl_2). The hydrolysis of Cl_2 is an equilibrium reaction ($K_1 = 1 \times 10^{-3}$ M^2) that yields the oxidant HOCl, Cl^-, and a proton, which combine to form HCl according to the reaction $Cl_2 + H_2O \leftrightarrow$

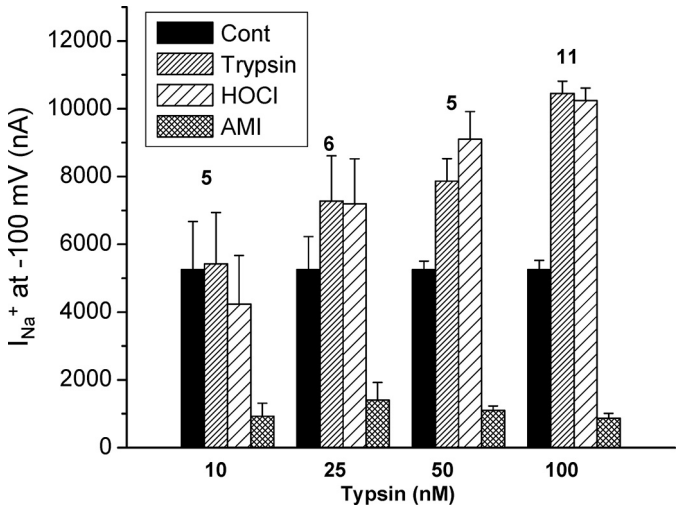

FIGURE 9. **Lack of inhibition of I_{Na} by chloramines after treatment of oocytes with various concentrations of trypsin.** hENaC-expressing oocytes were preincubated with the indicated concentrations of trypsin for 10 min and then perfused with 1 mM HOCl-ND96 for 10 min, at which time amiloride (*Ami*, 10 μM) was added in the bath (mean ± S.E.). *Cont*, control.

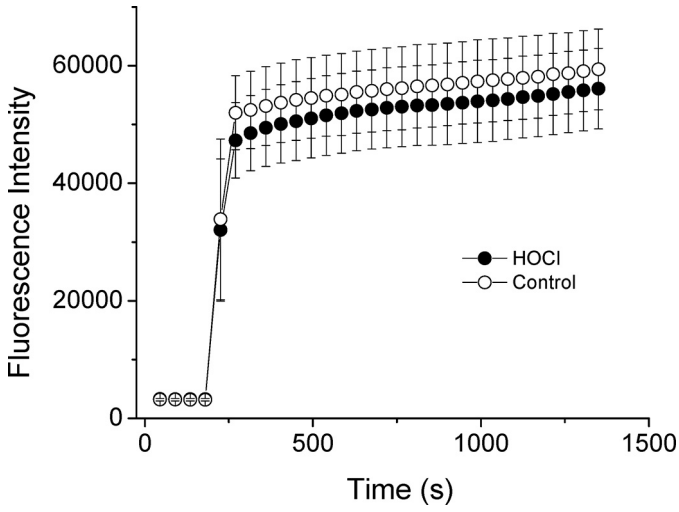

FIGURE 11. **Oocyte protease activity is not inhibited by HOCl.** hENaC-expressing oocytes were incubated with ND96 as control (○) or 2 mM HOCl-ND96 (●) for 10 min, then moved into a cuvette containing Boc-Gln-Ala-Arg-AMC-HCl (50 μM). Fluorescence was measured continuously for the next 20 min at 460 nm after excitation at 380 nm (mean ± S.E.; $n = 5$ for each condition).

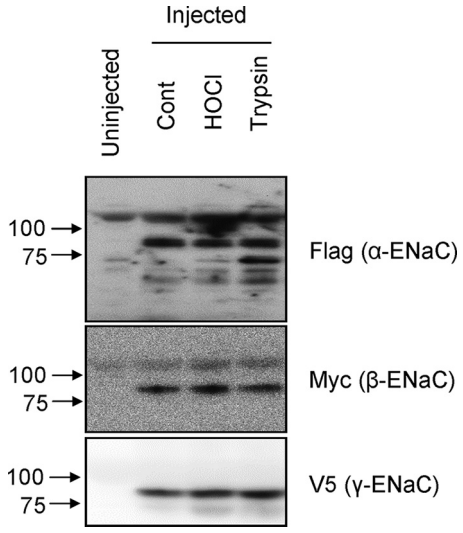

FIGURE 10. **HOCl does not decrease cell membrane ENaC levels.** *Xenopus* oocytes expressing tagged α-, β-, and γ-hENaC were incubated with ND96 (*Cont*) or 2 mM HOCl-ND96 (*HOCl*) for 10 min. In another set of experiments, hENaC-expressing oocytes were first incubated with trypsin (100 nM) for 10 min and then with 2 mM HOCl-ND96 (*Trypsin*). Equal amounts of membrane extracts from each treatment group were resolved by SDS-PAGE and blotted with anti-FLAG, Myc, and V5 antibodies to determine the levels of α-, β-, and γ-ENaC on the plasma membrane. Shown are the results of a typical experiment, which was repeated three times with identical results.

$HOCl + H^+ + Cl^-$. HOCl is a weak acid ($pK_a = 7.53$) and is, therefore, partly dissociated to OCl^- at physiological pH. Both HOCl and its conjugate base are also powerful oxidants. If one assumes the equilibrium depicted by this reaction is established in the epithelial lining fluid (pH 6.92, $[Cl^-] = 0.103$ M), one can calculate that $[OCl^-]/[Cl_2] = 2 \times 10^4$, and [OCl-]/[HOCl] = 0.25. Thus, in the airway lining fluid the concentration of HOCl will be ~80,000 that of Cl_2 at equilibrium. HCl damage to components of the alveolar epithelium is probably minimal due to the presence of a significant amount of bicarbonate (11 mM) in the epithelial lining fluid (54) that can neutralize it. Indeed, previous studies have shown that Cl_2 gas is much more toxic to the lung than aerosolized HCl (55).

Cl_2 *per se* also reacts with multiple organic compounds with fast kinetics (44), raising the question of which form (Cl_2 or HOCl) is responsible for the airway and lung injury during Cl_2 exposure (55). It should be stressed that although small concentrations of Cl_2 react with cellular components present in the upper airways (56, 57), at inhaled concentrations higher than 50 ppm (likely to be present during industrial accidents and terrorist attacks), Cl_2 will reach the distal airways and alveolar regions. Of course, low molecular weight scavengers (such as ascorbate and GSH) known to be present in high concentrations in the airway and epithelial lining fluid (for review, see Refs. 58 and 59) will react with Cl_2 and HOCl, reducing their steady state concentrations. However, our previous measurements and those of others show that the antioxidant scavenging capacity in the respiratory tract-lining fluid would be relatively limited compared with inhaled Cl_2 (19), and continuous exposure to a relative high concentration of Cl_2 gas (400 ppm for 30 min) should result in a high concentration of HOCl intermediates in airway lining fluid.

HOCl is a potent oxidant and will react with multiple biological molecules, including amines, to produce chloramines ($k \sim 10^5$ M^{-1} s^{-1}) (60). Thus, it is well accepted that in a cell culture system the observed biological effects of HOCl could be at least partially attributed to those of the chloramines depending on the relative abundance of HOCl and amines, as suggested by some reports (61, 62). Thus, organic chloramines, such as chloramines arising from the α amino group in free amino acids and the terminal amino group in peptides and proteins and the side chains of the amino acids histidine, tryptophan, and lysine, are likely to be formed *in vivo* (63–65). In addition, xenobiotic chloramines that may arise, for example, during water treatment using chlorination may also further contribute to the total exposure to organic chloramines. Our data showed that HOCl reacted with HEPES to form chloramines-type species, which are relatively stable and most likely responsible for the observed effects on ENaC. The fact that chloramines produced by the

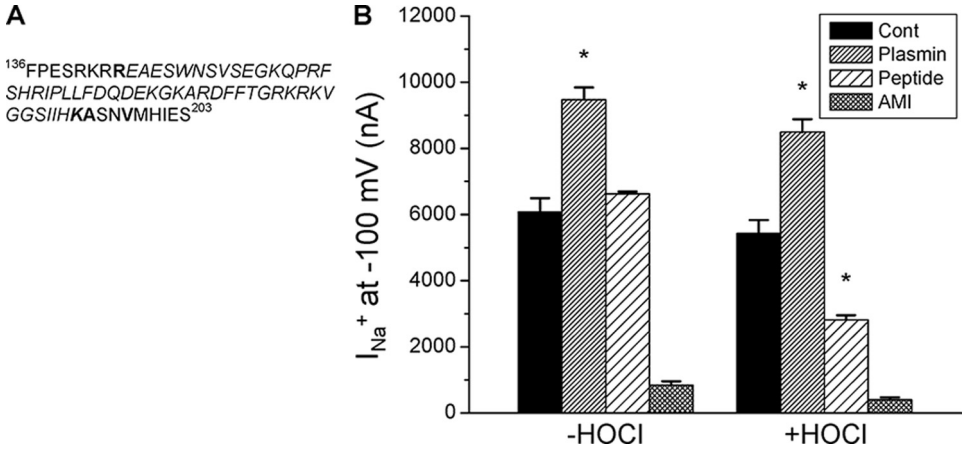

FIGURE 12. **HOCl inhibits ENaC by interaction with a peptide in the extracellular loop of γ-subunit.** *A*, shown is a partial sequence (136–203 amino acids) within the finger domain in the extracellular loop of γ-ENaC including the putative furin (Arg-143), plasmin (Lys-194), and elastase (Ala-195 and Val-198) cleavage sites (shown in *bold font*). A 51-mer peptide was synthesized corresponding to the segment between furin and plasmin cleavage sites (144–194, in *italics*). *B*, *left panel* (−*HOCl*), hENaC-expressing oocytes were perfused with plasmin (10 μg/ml) followed by ND96 containing the 51-mer peptide and then amiloride (10 μM). *Right panel* (+*HOCl*), after activation of hENaC by plasmin perfusion, oocytes were perfused with the mixture of 2 mM HOCl-ND96 and the peptide (3 μg/ml) (mean ± S.E.; $n = 6$ for each group; *, $p < 0.05$ compared with control). *Cont*, control.

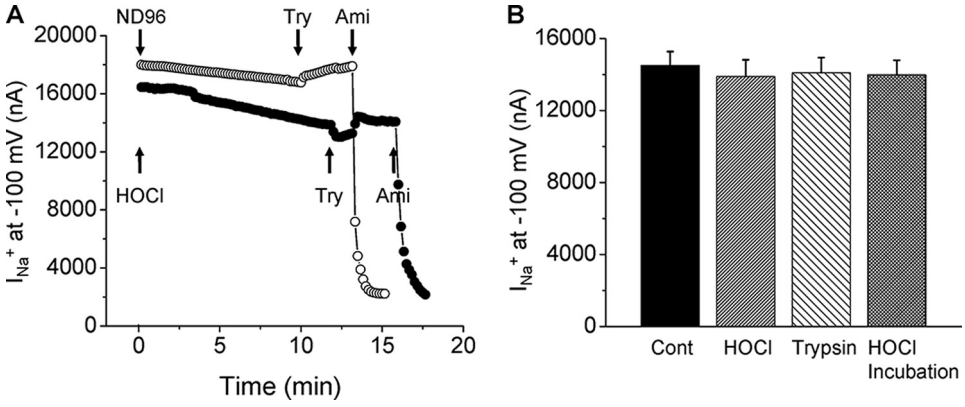

FIGURE 13. **I_{Na} of βS520K mutant ENaC is not inhibited by HOCl.** *A*, shown are representative time-course recordings of I_{Na} at −100 mV from oocytes expressing wild type α,βS520K and wild-type γ-ENaC perfused with HOCl-ND96 (1 mM) for 10 min (●) or incubated with HOCl-ND96 (100 μM) for 2 h (○), then perfused with trypsin (*Try*, 2 μM) and amiloride (*Ami*, 10 μM). *B*, group data showed neither perfusion nor incubation with HOCl-ND96 inhibits I_{Na} of βS520K mutant ENaC-expressing oocytes (mean ± S.E., $n = 14$ for HOCl perfused and 5 for HOCl incubated oocytes). *Cont*, control.

reaction of HOCl with glycine or taurine in the absence of HEPES also inhibit ENaC activity in the same manner as chloramines generated by the action of HOCl on HEPES further supports this conclusion. Of course it is probable that a number of other reactive intermediates may be formed that may also decrease ENaC activity.

The mechanisms by which chloramines inhibit ENaC are complex and poorly understood. Our data showed that plasma membrane ENaC levels were not decreased after exposure of oocytes to HOCl. It has been suggested that at least a protease uncleaved reserve pool of ENaC with low P_o and a furin-cleaved pool of ENaC with intermediate P_o exist at the cell membrane, and subsequent cleavage of γ-subunit distal to the furin site by protease further activates ENaC by increasing P_o (28, 29, 66, 67). Thus, the first question to be answered is which pool(s) of ENaC is affected by HOCl? Our data showed that both the inhibitions of amiloride-sensitive currents in oocytes after 10 min of exposure to HOCl and of alveolar fluid clearance of mice after exposure to Cl_2 were reversed by serine proteases. However, in both cases significant amounts of currents are still present after HOCl exposure. When oocytes were incubated in HOCl for long periods of time to the point at which amiloride-sensitive currents were decreased to almost zero, proteases failed to increase the sodium currents. In addition, I_{Na} of oocytes that was activated by exogenous proteases was no longer inhibited by HOCl. These results indicated that HOCl affected at least the ENaC pool with intermediate P_o, although ENaC channels in the high P_o pool that lacked the γ-subunit extracellular segment, because they had been further cleaved by proteases, were not sensitive to HOCl. One possible explanation for this insensitivity would be that HOCl damage on ENaC altered accessibility of cleavage sites to protease, similar to the regulation of proteolytic activation of ENaC by intracellular Na^+, as reported by Knight *et al.* (31).

It is known that ENaC undergoes complex post-translational modification, and protease cleavage has been shown to be important for ENaC activation (67). In our oocyte system, the extracellular loops of α- and γ-ENaC subunits were likely already cleaved by the endogenous protease furin; thus, the inhibitory segment in the α-subunit has been released, resulting in the observed single channel activity with intermediate P_o. When these oocytes were treated with exogenous proteases (trypsin, elastase, or plasmin), the proteolytic cleavage at the site distal to furin released another inhibitory segment in γ-subunit, resulting in additional activation of ENaC (48). The 51-mer peptide we synthesized represents the sequence between the putative furin and plasmin cleavage sites in the finger domain within the extracellular loop of γ-ENaC. Perfusion of oocytes with this peptide decreased the plasmin-activated currents, in agreement with what has been reported previously (68). Equally important, perfusion of plasmin-treated oocytes with both HOCl and the peptide resulted in a significant decrease of Na^+ currents, further demonstrating that the inhibitory effects of HOCl-derived chloramines and other intermediates on ENaC depends on this segment of γ-ENaC. Although the mechanism by which this segment confers the inhibition of ENaC by HOCl is not clear, recent reports have suggested a similar relationship

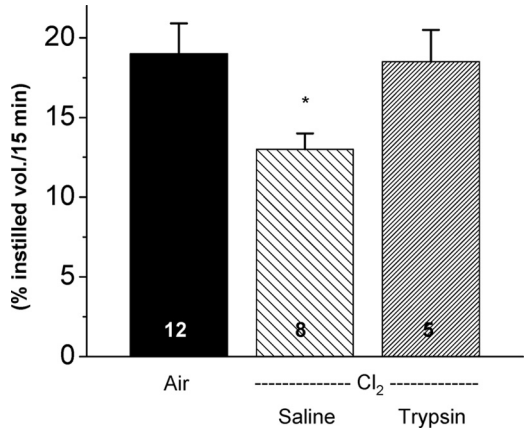

FIGURE 14. **Intratracheal trypsin reverses the decrease in AFC in mice exposed to Cl_2.** BALB/c mice were exposed to Cl_2 (300 ppm for 30 min) and then returned to room air. Fifteen minutes post-exposure, they were briefly anesthetized with isoflurane, and trypsin (5 μM; dissolved in 100 μl of saline) was instilled dropwise in the nostrils. Mice recovered quickly, and 1 h post-exposure, they were anesthetized and ventilated, and AFC was measured (mean ± S.E.; control = 12; saline = 8; trypsin = 5. *, $p < 0.05$).

between protease cleavage and extracellular or intracellular Na^+ inhibition of ENaC (31, 50). Thus, both proteases and HOCl regulated ENaC gating through action on the extracellular domain, so the locked-open βS520K mutant is not sensitive to either trypsin or HOCl.

It is not clear how HOCl chloramines affect ENaC gating. The extracellular loop of ENaC has been shown to be the binding site of ions or peptides modulating ENaC gating (69–71). It has also been suggested that the conformation of the γ-subunit segment within the thumb and finger domains in the extracellular loops (28) plays a critical role in the regulation of ENaC activity. One possibility is that the amino acid sequence in the 51-mer construct is important for the modulation of ENaC gating by HOCl. Interestingly, there is no cysteine residue in this sequence, which is suggested to be the amino acid most likely to be oxidized by HOCl (12, 72). Nevertheless, the possible chemical modifications imparted by HOCl to the 51-mer segment in ENaC can be assessed using the rate constants (60) for the reaction of HOCl with the side chain and the backbone amide nitrogen of the amino acids using a simple competition kinetics model for pseudo-first order conditions (large molar excess of the 51-mer over HOCl), as depicted in Table 1. The competition kinetics analysis indicates that histidines, modified to chlorohistidine, accounts for 83% of the chemical modifications, whereas modification of lysine residues to chlorolysine accounts for 13% of the chemical modifications. Tryptophan, modified to chlorotryptophan, accounts for 5% of the chemical modifications, whereas the rest of possible modifications occur with negligible yields. These amino acid chloramines may participate in chlorine transfer reactions or undergo hydrolysis to form carbonyl products.

Several studies have investigated the possible association between partially reduced reactive species and Na^+ transport across the alveolar epithelium in both animals and patients with acute lung injury. Reabsorption of isotonic fluid was inhibited during prolonged hemorrhagic shock. Instillation of aminoguanidine, an inhibitor of inducible nitric-oxide synthase, restored fluid reabsorption to normal levels (73). Mycoplasma infection

TABLE 1
Competition kinetics assessment of reactive centers in the 51-mer construct that are subject to chlorination by HOCl

Amino acid[a]	k[b]	n[c]	$n \times k$	P[d]
Arginine	26	5	1.3×10^2	5.4×10^{-4}
Asparagine	0.03	1	0.03	1.3×10^{-7}
Cysteine	3.0×10^7	0	0	0
Glutamine	0.03	2	0.06	2.5×10^{-7}
Histidine	1.0×10^5	2	2.0×10^5	0.83
Lysine	5.0×10^3	6	3.0×10^4	0.13
Methionine	3.8×10^7	0	0	0
Tryptophan	1.1×10^4	1	1.1×10^4	4.6×10^{-2}
Tyrosine	44	0	0	0
Backbone amide	10	50	5×10^2	2.1×10^{-3}

[a] Only those amino acids with $k \geq 0.03\ M^{-1}\ s^{-1}$ are listed here.
[b] Rate constant ($m^{-1}\ s^{-1}$) for the reaction of hypochlorite with the side chain of the amino acid or with the backbone amide nitrogen.
[c] Number of this amino acid present in the 51-mer or number of backbone amide nitrogens in the 51-mer.
[d] Normalized rate for reaction with HOCl calculated from the equation $P = n_i k_i / (\Sigma_i n_i k_i)$.

resulted in a significant decrease of both Na^+-dependent alveolar fluid clearance in BALB/c mice and inhibition of amiloride-sensitive Na^+ currents across alveolar type II cells isolated from these mice. However, normal levels of AFC were seen when BALB/c mice were pretreated with cyclophosphamide to suppress inflammatory cells and decrease production of reactive intermediates including HOCl (7). Reactive intermediates also inhibit vectorial Na^+ transport across ATII cells by decreasing the activity of epithelial Na^+ channels via post-translational modifications of either ENaC *per se* or of structural proteins (such as actin and fondrin) that are necessary for proper action of ENaC (6, 74). Substitution of a single tyrosine in the extracellular loop of αENaC (Tyr-283) with alanine prevented the peroxynitrite-induced decrease of amiloride-sensitive currents (75). ENaC was also inhibited by sulfhydryl-reactive agents added in the cytoplasm, and reducing agents reversed the run-down activity of ENaC in excised patches (76).

In summary, our combined *in vitro* and in *vivo* data demonstrate that chloramines and reactive intermediates formed by the interaction of Cl_2 and HOCl with the lung lining fluid and the incubation medium decrease amiloride-sensitive currents (a hallmark of ENaC) by the modulation of ENaC gating and that these effects can be prevented by serine proteases. These findings establish the rational basis for the use of small concentrations of serine proteases to prevent and counteract Cl_2-induced injury to epithelial sodium channels, thus, decreasing pulmonary edema.

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