Monochloramine Directly Modulates Ca^{2+}-Activated K^+ Channels in Rabbit Colonic Muscularis Mucosae

MADHU PRASAD,*,† JEFFREY B. MATTHEWS,* XUE D. HE,† and HAMID I. AKBARALI†

*Department of Surgery, Beth Israel Deaconess Medical Center, Boston; and †Research and Development, West Roxbury Veterans Affairs Medical Center, Harvard Medical School, Boston, Massachusetts

Background & Aims: Mesenteric ischemia, infection, and inflammatory bowel disease may eventuate in severe colitis, complicated by toxic megacolon with impending intestinal perforation. Monochloramine (NH_2Cl) is a membrane-permeant oxidant generated during colitis by the large amount of ambient luminal NH_3 in the colon. Reactive oxygen metabolites can modulate smooth muscle ion channels and thereby affect colonic motility, which is markedly impaired in colitis. ***Methods:*** Effects of NH_2Cl on ionic currents in the innermost smooth muscle layer of the colon, the tunica muscularis mucosae, were examined using the patch clamp technique. Membrane potential in whole tissue strips was measured using high-resistance microelectrodes. ***Results:*** Whole cell voltage clamp experiments showed that NH_2Cl (3–30 μmol/L) enhanced outward currents in a dose-dependent manner, increasing currents more than 8-fold at a test potential of +30 mV. Tail current analysis showed that the currents enhanced by NH_2Cl were K^+ currents. Inhibition by tetraethylammonium and iberiotoxin suggested that these currents represented activation of large-conductance, Ca^{2+}-activated K^+ channels. The membrane-impermeant oxidant taurine monochloramine, however, had no effect on whole cell currents. Single-channel studies in inside-out patches showed that NH_2Cl increased open probability of a 257-pS channel in symmetrical (140 mmol/L) K^+. In the presence of NH_2Cl, the steady-state voltage dependence of activation was shifted by −22 mV to the left with no change in the single-channel amplitude. The sulfhydryl alkylating agent *N*-ethylmaleimide prevented NH_2Cl-induced channel activation. NH_2Cl also hyperpolarized intact muscle strips, an effect blocked by iberiotoxin. ***Conclusions:*** NH_2Cl, at concentrations expected to be found during colitis, may contribute to smooth muscle dysfunction by a direct oxidant effect on maxi K^+ channels.

Impaired colonic motility contributes significantly to the morbidity of colitis associated with ischemia, infection, and inflammatory bowel disease.[1–5] Severe colitis may lead to toxic megacolon, a condition in which transmural colonic inflammation results in a progressively dilated and aperistaltic segment of involved colon at risk of perforation. The mechanisms underlying colonic dysmotility in colitis are unclear but may involve changes at the neuromuscular junction arising from alterations in enteric neurotransmission pathways, in smooth muscle cell contractility, or both.[6–8] Additionally, the abnormal slow wave amplitude and duration present during colitis are accompanied by ultrastructural changes in interstitial cells of Cajal.[9,10] The potential role of local inflammatory mediators, in particular free radical oxidants, on smooth muscle cells is not well understood.

Under physiological conditions, bacteria ferment nitrogen-containing compounds in the lumen of the large intestine to produce large amounts of ammonia (NH_3/NH_4^+) locally (10–70 mmol/L luminal), creating a unique microenvironment within the colon.[11] The pK_a of NH_3 is 9.3; therefore, at physiological pH it exists predominantly as the protonated species, NH_4^+. Destruction of the epithelial barrier in colitis can result in significant accumulation of NH_4^+ within the lamina propria. Monochloramine (NH_2Cl) is a highly potent lipophilic oxidant produced during colitis when activated neutrophils (polymorphonuclear leukocytes [PMNs]) infiltrate the bowel wall, releasing hydrogen peroxide (H_2O_2) and the enzyme myeloperoxidase (MPO) into the extracellular milieu.[12] MPO catalyzes the reaction of H_2O_2 with ambient Cl^- ions to form the toxic chlorinated oxidant, hypochlorous acid (HOCl).[13]

$$H_2O_2 + Cl^- \xrightarrow{MPO} HOCl + H_2O$$

Abbreviations used in this paper: EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; IBTX, iberiotoxin; MPO, myeloperoxidase; NEM, *N*-ethylmaleimide; PMN, polymorphonuclear leukocyte; SH, sulfhydryl; TEA, tetraethylammonium; TMM, tunica muscularis mucosae.

0016-5085/99/$10.00

The large quantities of NH_4^+ present in the colon (10–70 mmol/L luminal) are rapidly converted by HOCl into NH_2Cl.[14]

$$NH_4^+ \rightleftarrows NH_3 + H^+$$

$$HOCl + NH_3 \rightarrow NH_2Cl + H_2O$$

NH_2Cl is a potent oxidizing agent because it contains Cl^+, the oxidized chlorine atom.[12,15] In the presence of NH_4^+, PMN-related acute tissue damage is mediated largely by NH_2Cl.[16–18] Because it is lipophilic and rapidly penetrates cell membranes, NH_2Cl is continually depleted from its site of production in the extracellular space, thereby favoring its continued production by mass action.[12,15,19,20] NH_2Cl is difficult to measure in vivo because it enters cells so easily; however, it has been estimated that local concentrations of 200–300 μmol/L are typically present during severe colitis.[21]

Several studies have suggested that NH_2Cl may be a particularly important mediator in colitis, stimulating fluid and electrolyte secretion and contributing to local tissue damage.[22,23] Certain oxidizing agents are also known to modulate smooth muscle function; however, the effects of NH_2Cl on colonic smooth muscle and its influence on colonic motility have not been previously addressed.

Phasic contractions of the colon are mediated by action potentials superimposed on slow wave activity.[24] The summation of a series of action potentials results in spike-dependent contraction. It is well known that in colitis, the contractile ability of colonic smooth muscle is markedly impaired.[3,7,8] Ion channels regulate the electrical activity of smooth muscle; thus modulation of ionic currents in smooth muscle is a potential mechanism by which inflammatory mediators can affect colonic motility. The aim of this study was to examine the effect of NH_2Cl on ionic currents in smooth muscle cells from the tunica muscularis mucosae (TMM) of the rabbit distal colon, the smooth muscle layer adjacent to the mucosa. We show that concentrations of NH_2Cl within the range encountered in colitis irreversibly activate the large-conductance Ca^{2+}-activated (maxi) K^+ channel in a calcium-independent fashion, likely involving modification of sulfhydryl (SH) groups on the channel itself. This effect may contribute to the motor dysfunction encountered in severe colitis.

Materials and Methods

Cell Dispersal

Single smooth muscle cells of the rabbit colonic muscularis mucosae were prepared as described previously.[24] Briefly, New Zealand white rabbits (2–2.5 kg) were anesthetized with intramuscular ketamine (12.5 mg/kg) and then killed by intravenous injection of pentobarbital (32 mg/kg). Laparotomy was performed, a segment of the distal colon was removed, and its lumen was flushed with saline to remove fecal pellets. The colon was opened longitudinally along its mesenteric aspect and pinned with its mucosa side down on a Sylgard-coated (Dow Corning, Midland, MI) Petri dish that contained low-Ca^{2+} Tyrode's solution gassed with 95% O_2/5% CO_2. The muscularis externa was removed by sharp dissection under a stereo microscope, and the underlying submucosa and muscularis mucosae were carefully separated from the mucosa using fine forceps. After the muscularis mucosae had been divided into small strips, it was incubated for 20 minutes at room temperature in low-Ca^{2+} Tyrode's solution containing 0.25 mg/mL collagenase, 0.3 mg/mL trypsin, and 1 mg/mL bovine serum albumin. The tissue was gently triturated with a wide-bore, fire-polished Pasteur pipette, and then transferred into an enzyme-free solution where trituration was continued to facilitate release of individual spindle-shaped myocytes. For experiments, cells were placed in a recording chamber on the stage of an inverted, phase-contrast microscope (Olympus CK-2; Olympus, Tokyo, Japan) and allowed to settle before being perfused by solutions carried by a gravity-driven perfusion system.

Whole tissue microelectrode studies were performed in intact segments of the rabbit distal colon. The serosa and outer longitudinal muscle layer were removed sharply to facilitate introduction of microelectrodes into the circular muscle layer.

Solutions

The low-Ca^{2+} Tyrode's solution contained (in mmol/L) NaCl, 137; KCl, 2.7; NaH_2PO_4, 0.36; $NaHCO_3$, 12; $CaCl_2$ (10 μmol/L); and glucose, 5.5. The Krebs' solution was composed of (in mmol/L) NaCl, 115; KCl, 4.6; NaH_2PO_4, 1.2; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 21.9; and glucose, 11.5. This was gassed with 95% O_2/5% CO_2. During whole cell recordings, the chamber was perfused with a solution buffered with HEPES containing (in mmol/L) NaCl, 135; KCl, 5.4; NaH_2PO_4, 0.33; HEPES, 5; $MgCl_2$, 1; $CaCl_2$, 2; and glucose, 5.5 (pH 7.2 with 1N NaOH). This was gassed with 100% O_2. During tail current measurement, varying extracellular K^+ concentrations were used to replace equimolar Na^+. Whole cell recordings were made with pipettes containing (in mmol/L) Kaspartate, 100; KCl, 30; $MgCl_2$, 2; ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 5; adenosine triphosphate, 5; and guanosine triphosphate, 0.1. The pH was adjusted to 7.2 with KOH. Some experiments were performed with 5 mmol/L 1,2-bis(2-aminophenoxy)-ethane-*N,N,N′,N′*-tetraacetic acid instead of EGTA.

For single channel recordings in inside-out patches, the Ca^{2+}-free bath solution contained (in mmol/L) KCl, 140; HEPES, 10; EGTA, 10; $MgCl_2$, 1.2; and glucose, 10. Solution pH was adjusted to 7.4 with 10N KOH. Under these conditions, the free Ca^{2+} concentration is <1 nmol/L. This low concentration of Ca^{2+} was set to study voltage-dependent channel behavior. Single channel recordings were made with a

pipette solution containing (in mmol/L) KCl, 140; EGTA, 10; HEPES, 5; and $MgCl_2$, 2 (pH 7.4 with 10N KOH). In some experiments, 20 nmol/L iberiotoxin (IBTX), an external blocker of the large-conductance Ca^{2+}-activated K^+ channel, was included in the pipette solution. Steady-state voltage dependence of activation was determined using a bath solution containing (in mmol/L) KCl, 140; HEPES, 10; EGTA, 1; $CaCl_2$, 0.61; and glucose, 10 (pH 7.4 with 10N KOH). The effective free Ca^{2+} concentration is 100 nmol/L under these conditions.

Preparation of NH_2Cl

NH_2Cl was synthesized by the dropwise addition of 200 μL NaOCl (500 mmol/L) to a solution containing 10 mL NH_4Cl (20 mmol/L) and Na_2HPO_4 (5 mmol/L) at 0°C. Beer's law was used to calculate the concentration of NH_2Cl in the stock solution by measuring the solution absorbance at 242 nm and using a molar extinction coefficient of 429 for NH_2Cl at this wavelength.[25] Stock solutions prepared in this manner reliably contained 2.2–2.5 mmol/L NH_2Cl. Taurine monochloramine (taurine NHCl) was prepared in a similar manner using a solution containing 20 mmol/L taurine instead of NH_4Cl. The concentration of taurine NHCl in the stock solution was assayed by measuring its characteristic absorbance at 252 nm. During experiments, stock solutions of NH_2Cl were diluted in buffered solutions, bathing cells, or patches to achieve the concentration indicated (1–30 μmol/L). NH_2Cl did not alter solution pH. Control experiments performed with either NaOCl or the NH_4Cl/Na_2HPO_4 solution alone did not emulate the changes induced by NH_2Cl.

Recording Techniques

Standard gigaseal patch clamp technique was used to make recordings in either whole cell configuration or in inside-out patches.[26] The Axopatch 200B patch clamp amplifier (Axon Instruments, Foster City, CA) was used in all experiments. Recordings were made using microelectrodes fashioned from borosilicate glass (1.00-mm internal diameter; World Precision Instruments, Sarasota, FL) using a Flaming/Brown type horizontal pipette puller (Sutter Instruments, Novato, CA) and fire-polished on a microforge (MF-83; Narishige, Tokyo, Japan). The resistance of filled pipettes used for whole cell and inside-out patch recordings was 3–5 MΩ. Single-channel recordings were made with Sylgard-coated pipettes. All recordings were made at room temperature (22–24°C). Microelectrodes used for intact tissue recordings had tip resistances of 30–80 MΩ. Whole tissue studies were performed at 30°C.

Membrane currents were digitized with a Digidata 1200B analog-to-digital convertor controlled by pClamp 7.0 software (Axon Instruments). Data were displayed online during experiments and stored on the hard disk of a desktop computer (Hewlett Packard Vectra XA, Eybens, France). During whole cell experiments, currents were sampled at 2 kHz and filtered at 1 kHz. Single-channel events were sampled at 20 kHz and filtered at 5 kHz. Unitary current amplitudes were measured using on-screen cursors. Brief channel openings of <1 millisecond duration were ignored for amplitude measurements. Series resistance was not compensated in these studies.

All inside-out patches examined contained more than 1 channel. Open probability is therefore expressed as NP_o, and was determined from the expression $P_o = I_{mean}/iN$, where I_{mean} is the mean current measured in data records and i is the single channel amplitude. The mean current was obtained by averaging the current responses to several slow (2.5-second) voltage ramps applied to each patch.[27] This was compared with the mean current calculated from amplitude histograms of single-channel records using the expression $I_{mean} = A_1f_1 + A_2f_2 + A_3f_3 + \ldots + A_nf_n$, where A_1, A_2, and A_n represent the area under the gaussian curve for each current level (f_1, f_2, . . ., f_n) present in the patch. There was excellent agreement between the 2 techniques of calculation.

Membrane potential was recorded in segments of rabbit distal colonic circular muscle using high-resistance microelectrodes. Tissues placed in an Abe-Tomita chamber were continuously perfused with oxygenated Krebs' solution at 30°C. Recordings were made under nonadrenergic, noncholinergic conditions with atropine (1 μmol/L) and guanethidine (3 μmol/L) included to suppress cholinergic- and adrenergic-mediated excitatory responses, respectively. A desensitizing concentration (1 μmol/L) of substance P was used to block the substance P–mediated excitatory junction potential. Electrical field stimulation consisted of 4 pulses of 50 mV at 20 Hz for 0.5 milliseconds. The inhibitory junction potentials intermittently evoked by this electrical field stimulation confirmed tissue viability. Stimulation of intramural nerves was accomplished with a stimulator (Grass S-88; Grass Instruments, Quincy, MA) in series with a stimulus isolation unit (Grass SIU) and a constant current unit (Grass CCU 1). Data are expressed as mean ± SEM.

Chemicals

Chemicals were reagent grade and purchased from either Sigma Chemical Co. (St. Louis, MO) or from Fluka (Ronkonkama, NY). IBTX was obtained from Bachem Bioscience (King of Prussia, PA). Collagenase was from Yakult (Tokyo, Japan).

Results

Whole Cell Experiments

In current clamp, the average resting potential of isolated cells hyperpolarized by 13.0 ± 1.2 mV on exposure to 10 μmol/L NH_2Cl (n = 9). In the first set of voltage clamp experiments, myocytes were studied in conventional whole cell configuration. Cells were maintained at a holding potential of −50 mV and then stepped in 10-mV increments from −70 mV to + 30 mV, resulting in a family of outward currents (Figure 1*A*). After exposing the cells to 10 μmol/L NH_2Cl for 5 minutes, the magnitude of outward currents increased markedly (Figure 1*B*). At 10 μmol/L NH_2Cl, currents increased more than 8-fold, from 162 ± 21 to 1346 ±

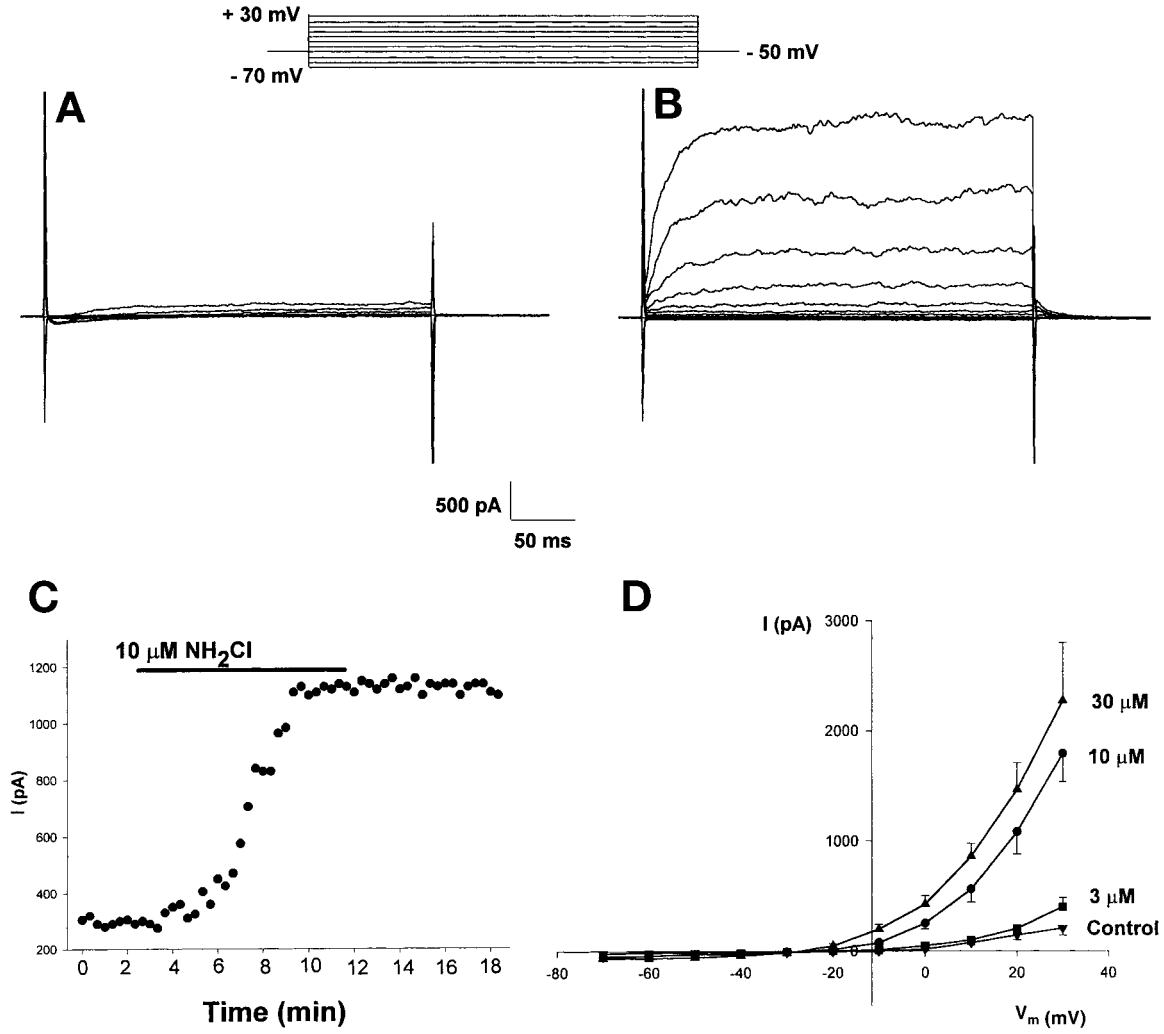


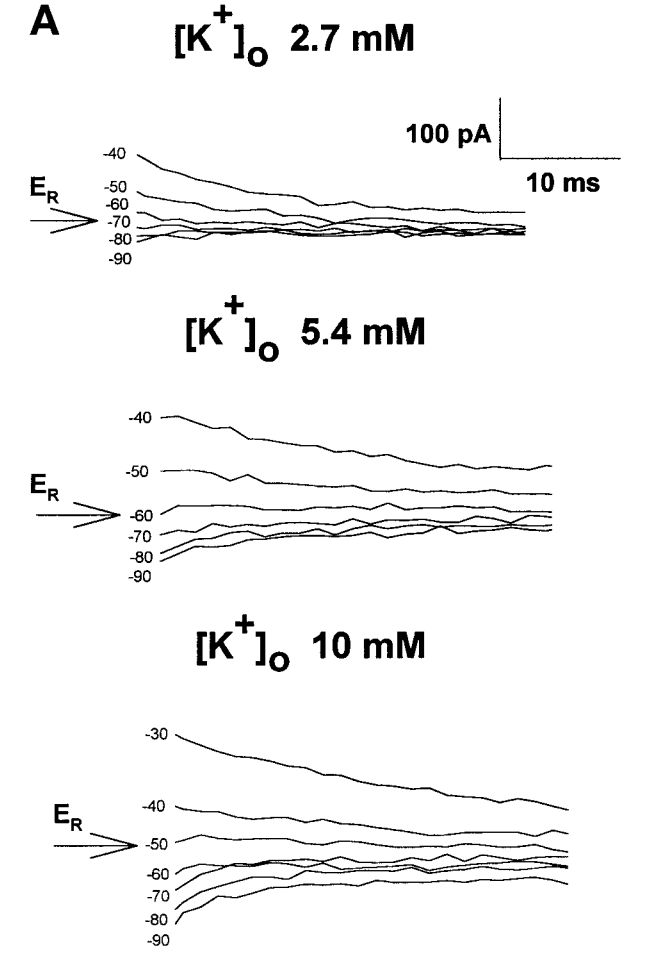
Figure 1. Effect of NH_2Cl on outward currents. Conventional whole cell recordings of membrane currents in cells dialyzed with 130 mmol/L K^+ and 10 mmol/L EGTA. (*A*) Family of small voltage–dependent outward currents generated by applying the protocol shown. (*B*) Enhancement of voltage-dependent outward currents after exposure to 10 μmol/L NH_2Cl for 5 minutes. (*C*) Time course of NH_2Cl effect. Currents begin to increase after perfusion with NH_2Cl for 1 minute, with peak effect observed after 5 minutes. Enhanced currents are irreversible. (*D*) Dose dependence of NH_2Cl effect. Peak effects observed at [NH_2Cl] > 10 μmol/L. Enhanced currents are outwardly rectifying. Capacity transients were not canceled in these and subsequent recordings.

109 pA (n = 15), at a test potential of +30 mV. NH_2Cl-induced outward currents peaked after 5 minutes and did not reverse after washout (Figure 1*C*). Enhanced currents persisted for over 30 minutes. NH_2Cl activated outwardly rectifying currents in a dose-dependent manner (Figure 1*D*). The threshold of activation occurred at 3 μmol/L, with a peak effect at 30 μmol/L. Higher concentrations of NH_2Cl (100 μmol/L) did not further enhance the magnitude of outward currents.

To identify the nature of the outward currents, the reversal potential of tail currents at 3 different concentrations of external K^+ was measured in the presence of NH_2Cl and plotted against log [K^+] (Figure 2). The slope of this line (51 mV/decade) agreed with the value predicted by the Nernst equation for a pure K^+ conductance (57 mV/decade), indicating that the outward currents enhanced by NH_2Cl reflected opening of a K^+ conductance.

NH_2Cl-induced activation of K^+ currents was abolished by 10 mmol/L external tetraethylammonium (TEA) (Figure 3). Pretreatment of cells with TEA prevented NH_2Cl enhancement of outward currents (Figure 3*B*); however, enhanced currents were unmasked after TEA washout. At a test potential of +50 mV, NH_2Cl-enhanced current (100% control, n = 8 cells) was not inhibited by 3 mmol/L 4-aminopyridine (114% ± 1.5% control, n = 4), which blocks many smooth muscle delayed rectifier K^+ channels (K_{DR}), or apamin (300 nmol/L), which blocks the small-conductance Ca^{2+}-activated K^+ channel (SK) (110% ± 10% control, n = 4) (Figure 4).

To determine whether the effects of NH_2Cl were



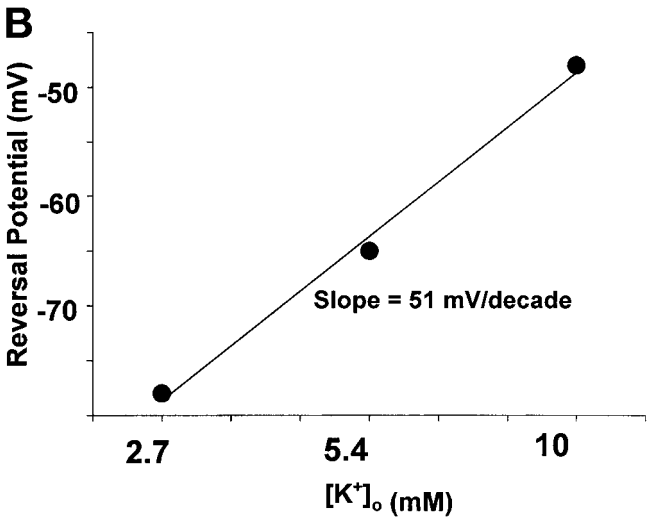


Figure 2. Tail current analysis. Cells held at −50 mV were depolarized to +50 mV for 200 milliseconds and then clamped to various potentials. (*A*) Potential at which tail current reversal observed when cells were perfused with different external K^+ concentrations. (*B*) Plot of reversal potential against log $[K^+]_o$ reveals a Nernstian relationship.

mediated via internalization of the compound, we examined the effects of taurine-NHCl, a large, negatively charged NH_2Cl analogue that does not permeate the plasma membrane.[23] Taurine-NHCl (100 μmol/L) had no

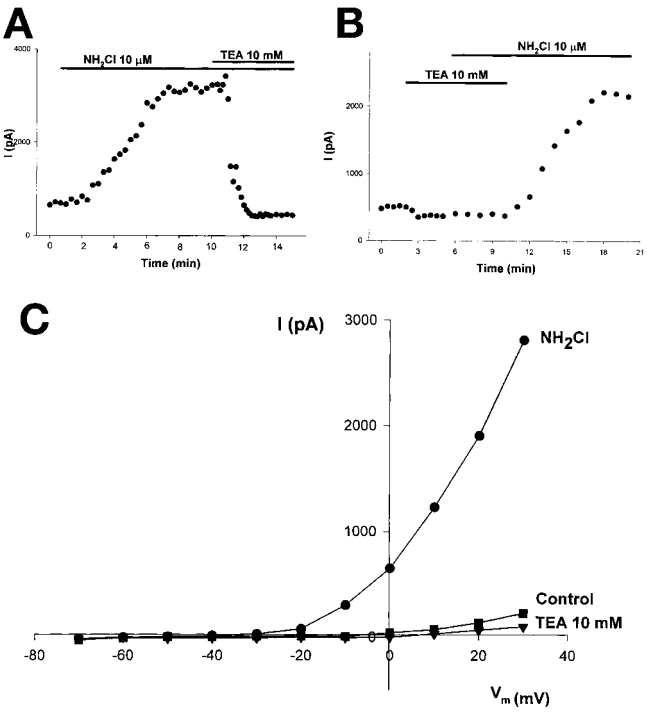


Figure 3. Effect of TEA on NH_2Cl activated currents. (*A*) Time course over which TEA (10 mmol/L) inhibits NH_2Cl-stimulated currents at a holding potential of +50 mV. (*B*) Preexposure to TEA prevented the NH_2Cl-induced increase in outward currents. TEA washout unmasked NH_2Cl effect. (*C*) I–V (current–voltage) relationship showing reversal of NH_2Cl effect by external TEA.

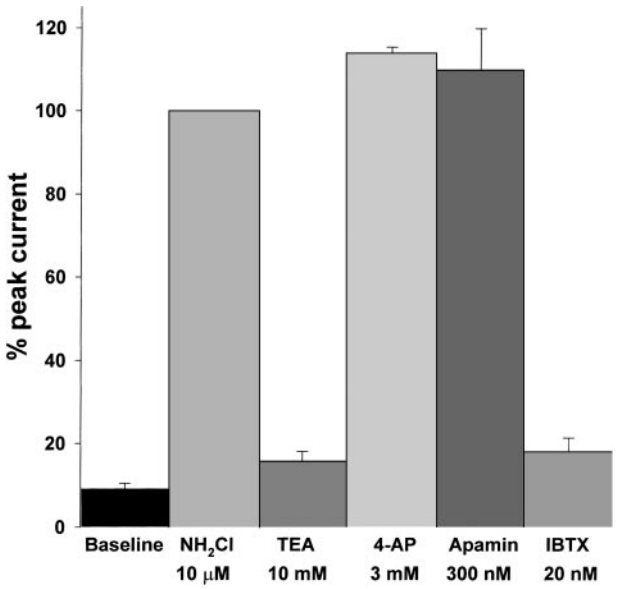


Figure 4. Effect of K^+ channel blockers on NH_2Cl-activated currents. Peak currents were measured at +50 mV test potential. Currents in the presence of NH_2Cl were taken as 100%. Control current was markedly enhanced by 10 μmol/L NH_2Cl and inhibited by TEA. Neither 4-AP nor apamin inhibited the NH_2Cl activated currents. Data represent mean ± SEM for 4 cells.

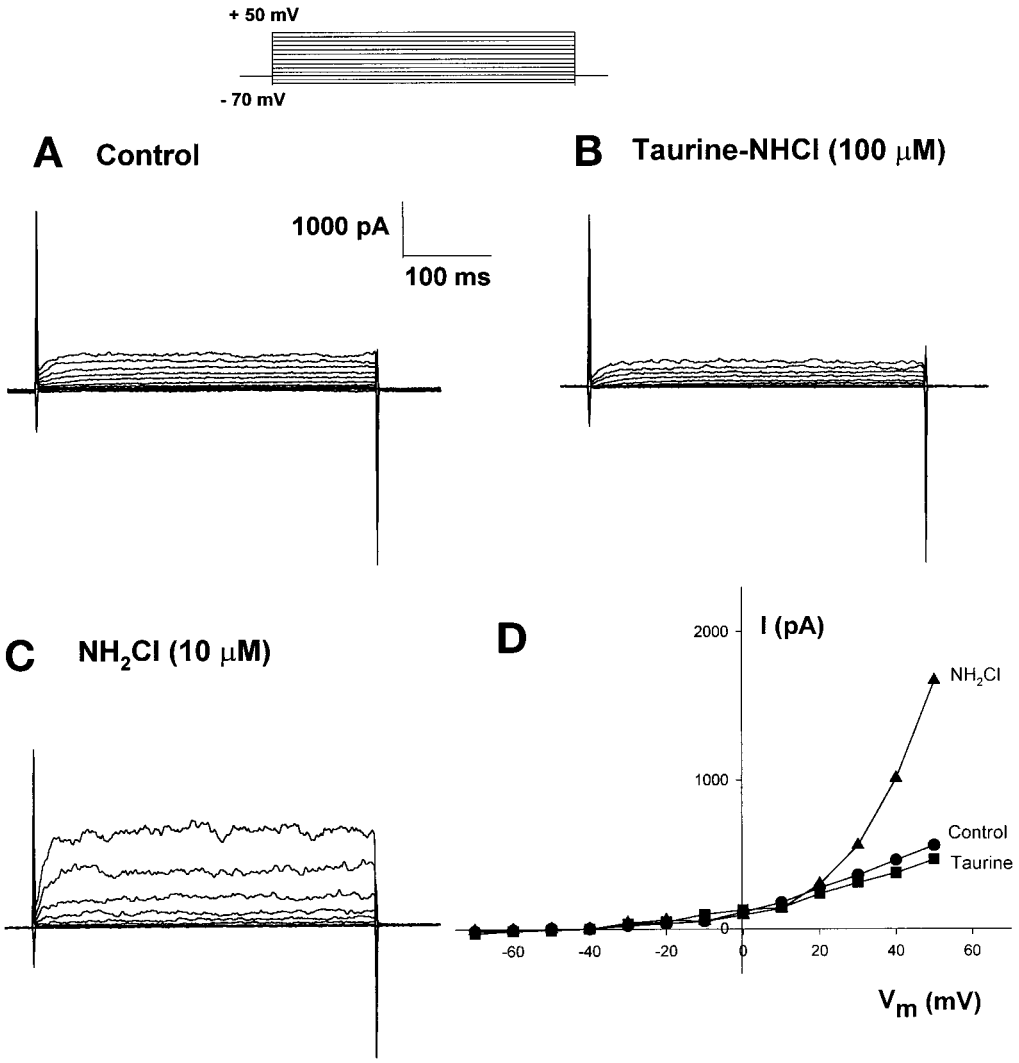


Figure 5. Effect of the membrane-impermeant NH_2Cl analogue, taurine NHCl, on whole cell outward currents. Voltage protocol shown was applied. (*A*) Family of outward currents before perfusion with taurine NHCl. (*B*) Taurine NHCl has no effect on control currents. (*C*) When the same cell is exposed to NH_2Cl, outward currents are activated. (*D*) I–V relationship comparing the effect of NH_2Cl and taurine NHCl on outward currents.

effect on outward currents (226 + 37 pA [control] vs. 207 + 29 pA, n = 4; Figure 5). However, when the same cell was subsequently exposed to NH_2Cl (10 μmol/L), K^+ currents could be activated (914 + 88 pA, n = 4), showing that NH_2Cl exerts its effects on the TEA- and IBTX-sensitive K^+ channel after crossing the plasma membrane and entering the cytosol.

Inside-out Patches

To further elucidate the mechanism by which cytosolic NH_2Cl activated the TEA-sensitive K^+ channel and to more fully characterize the K^+ channel, single-channel studies were performed in inside-out patches. In the presence of high cytosolic Ca^{2+} buffering, large-conductance single-channel events were largely voltage dependent and reversed at 0 mV. The single-channel conductance was estimated to be 257 ± 10 pS in symmetrical 140-mmol/L K^+ solutions. Application of NH_2Cl (6 μmol/L) to the cytosolic side of the patch resulted in a marked increase in the activity of the large-conductance channel (Figure 6*A* and *B*). The open probability (NP_o) of the channel increased from 0.21 to 0.71 at a holding potential of +40 mV after exposure to NH_2Cl. The increase in NP_o was not caused by modification of the single-channel amplitude (Figure 6*C* and *D*).

Voltage ramps were applied to inside-out patches, and the mean current was calculated from 10 such superimposed ramps. NH_2Cl increased mean current in inside-out patches (Figure 7*C*). When 20 nmol/L IBTX, a highly specific blocker of the large-conductance Ca^{2+}-activated (maxi) K^+ channel, was included in the patch pipette, control current as well as NH_2Cl-activated current were both markedly attenuated (Figure 7*C*), showing that NH_2Cl enhanced the activity of the maxi K^+ channel. Channel activity was unaffected by internal TEA (10 mmol/L).

The voltage dependence of channel activation was examined in the absence and presence of NH_2Cl. Each curve was fitted to a Boltzmann relationship of the form:

$$NP_{open} = n/[1 + \exp[-K(V - V_{1/2})]]$$

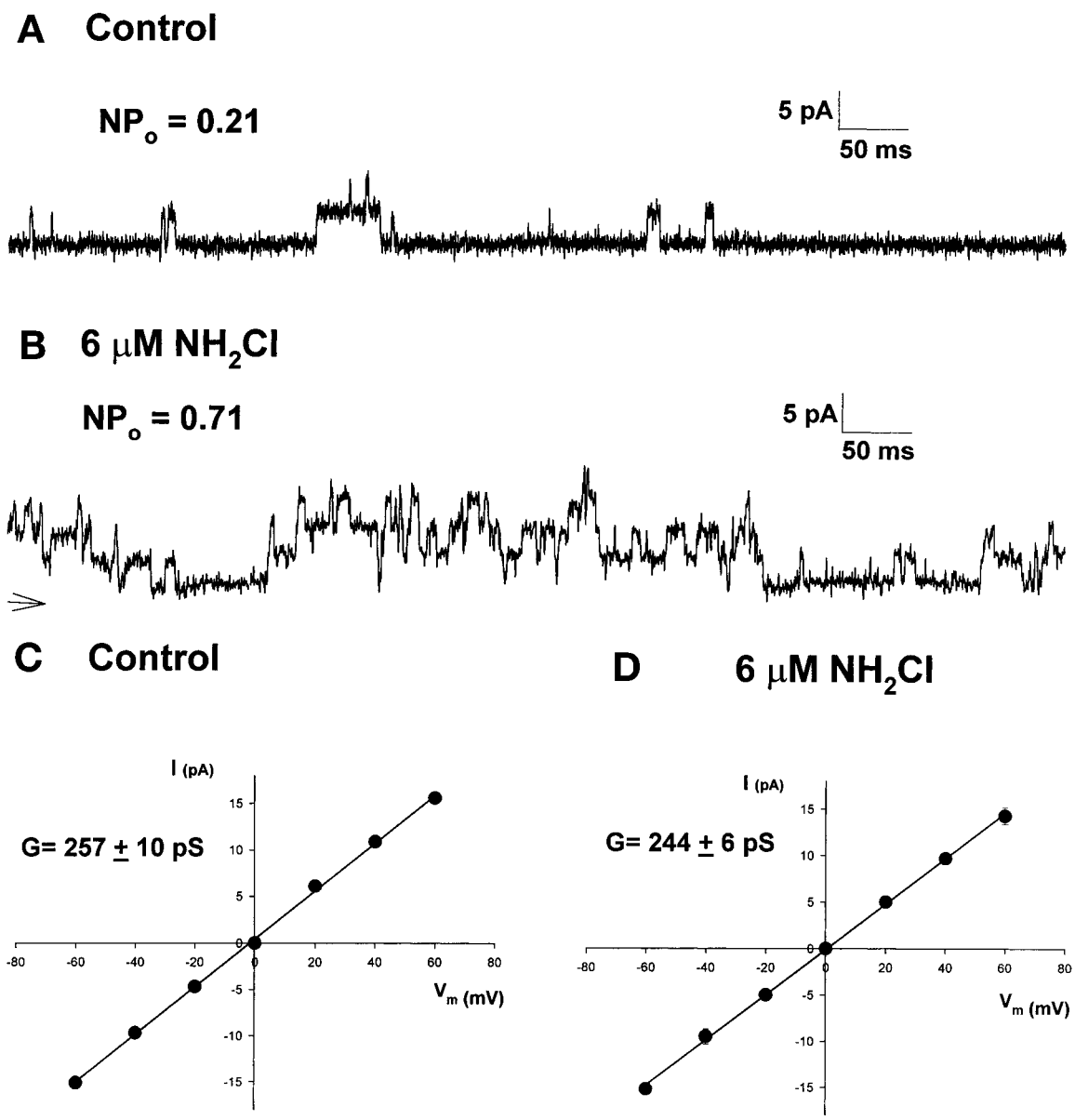


Figure 6. Effect of NH_2Cl on single-channel activity of inside-out patches. Test potential, +20 mV. (*A*) Basal channel activity. Intermittent opening of a large-conductance channel is observed. (*B*) Channel activity is markedly enhanced in a patch exposed to NH_2Cl (6 μmol/L) for 5 minutes. NP_o increased from 0.21 (control) to 0.71 (NH_2Cl). (*C*) I–V relationship shows that channel conductance is 257 pS. (*D*) After exposure of patches to NH_2Cl, single-channel amplitude does not change. Data represent mean ± SEM for 6 patches.

where N is the number of channels in the patch, n is the maximal NP_{open}, K^{-1} is the steepness of the voltage-dependent activation, and $V_{1/2}$ is the voltage of half-maximal activation. The activation curve was shifted to a more negative potential in the presence of NH_2Cl with a shift in the $V_{1/2}$ from 107 to 85 mV (Figure 8) with no change in the slope factor, K^{-1} (20.3, control vs. 18.9, 6 μmol/L NH_2Cl).

Redox reactions frequently result in the modification of amino acid SH groups. Agents that alkylate SH groups prevent subsequent modification of these moieties by oxidants. When the cytosolic face of inside-out patches was treated with 1 mmol/L *N*-ethylmaleimide (NEM), mean current was reduced (Figure 9*A* and *B*). In addition, NEM pretreatment eliminated channel activation by NH_2Cl and prevented increases in I_{mean} (Figure 9*B*). After 1 hour of exposure, NEM completely abolished channel activity and NH_2Cl-induced current activation. These data show that NH_2Cl-induced activation of the maxi K^+ channel in myocytes of the rabbit colonic muscularis mucosae results from SH group oxidation.

Whole Tissue Studies

Membrane potential recordings were made in strips of intact circular muscle from the rabbit distal colon. Under nonadrenergic, noncholinergic conditions,

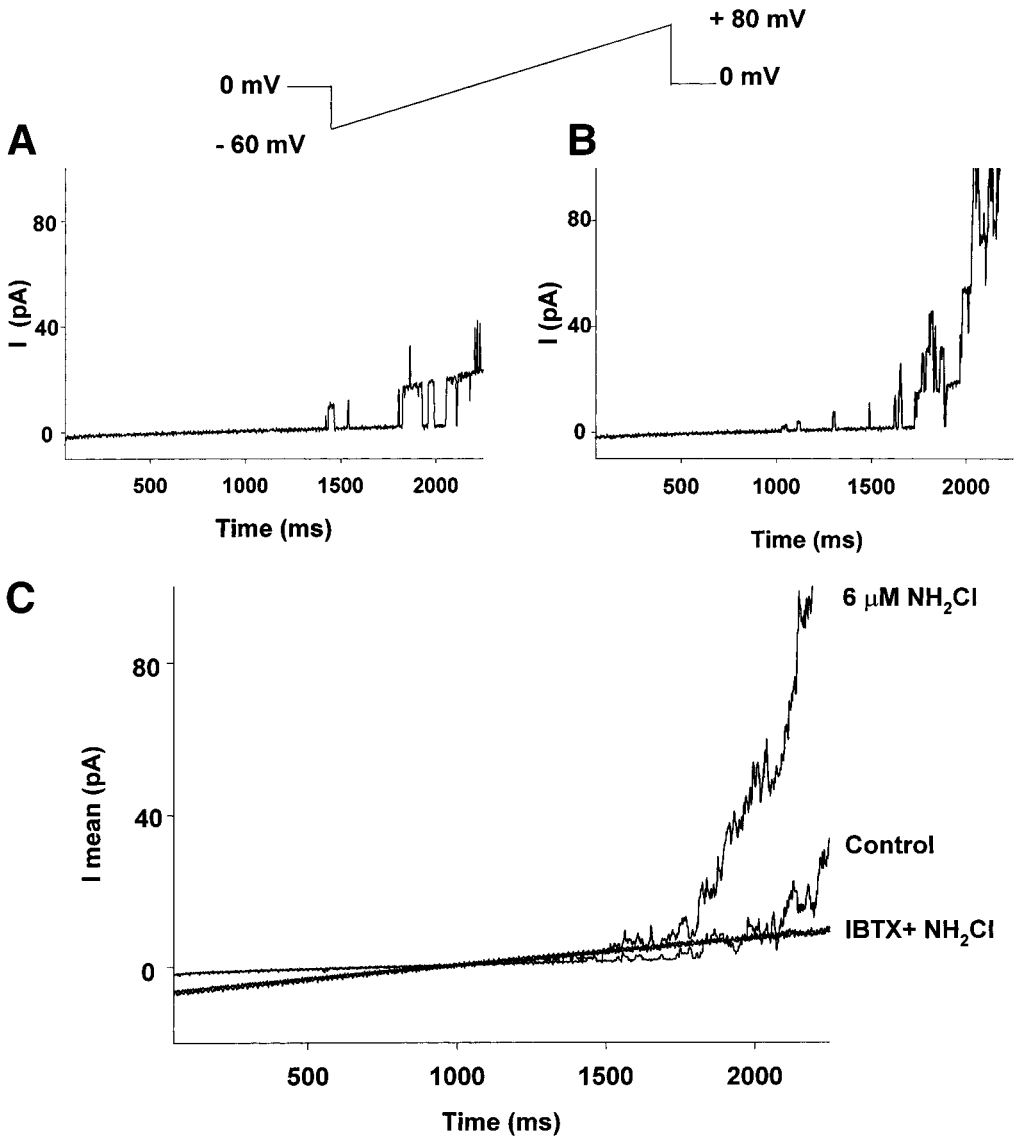


Figure 7. Effect of NH_2Cl on mean current in inside-out patches. Voltage ramps were applied as shown to inside-out patches at a rate of 0.05 mV/ms. (*A*) Tracing exhibiting current response to a single ramp before perfusion with NH_2Cl. Individual channel openings are observed at positive potentials, and single-channel amplitude increases at more positive potentials. (*B*) Tracing of current response to a single voltage ramp in the patch after exposure to 6 μmol/L NH_2Cl for 5 minutes. Channel opening is observed at potentials less positive than in control patch. Peak current after NH_2Cl treatment is higher than control at potentials shown. (*C*) Mean current in inside-out patch before and after treatment with NH_2Cl. Each curve represents the average of current response to 10 superimposed voltage ramps as in *A* or *B*. NH_2Cl markedly enhances mean outward patch current. The lower trace shows, in a separate patch, that the channel activity observed with control or NH_2Cl-treated patches is abolished when 20 nmol/L IBTX is included in the patch pipette.

exposure to NH_2Cl hyperpolarized the circular muscle by 11 mV, confirming the result in single cells (Figure 10). This effect was irreversible. In tissues preincubated with the highly specific maxi K^+ channel blocker IBTX, NH_2Cl failed to hyperpolarize cells. This suggests that hyperpolarization induced by NH_2Cl results from activation of maxi K^+ channels.

Discussion

In this report we show that NH_2Cl, a long-lived, highly reactive product of the respiratory burst, activates the large-conductance Ca^{2+}-activated (maxi) K^+ channel in colonic smooth muscle. Activation of the maxi K^+ channel by NH_2Cl occurs in a Ca^{2+}-independent manner and results from direct modification of SH residues on the channel protein. In addition, NH_2Cl hyperpolarizes the smooth muscle cells. Microelectrode recordings from whole tissue strips also show a hyperpolarization of 11 mV, which is prevented by preincubation with IBTX. This suggests that the effect of NH_2Cl on membrane potential is mediated by maxi K^+ channels. Because NH_2Cl is synthesized in large amounts in acute colitis of diverse etiology, it may contribute to dysmotility observed during colitis by altering resting membrane potential and repolarization of the action potential, processes largely regulated by K^+ channel activity. Hypomotility present during colonic inflammation leads to impaired segmentation contractions, suboptimal contact of the epithelium with the fecal stream, and the diarrhea that accompanies colitis, contributing further to the pathophysiology associated with this condition.

In voltage clamp, using conventional whole cell gigaseal patch clamp recording techniques, exposure of rabbit colonic smooth muscle to NH_2Cl led to the irreversible activation of large, outwardly rectifying currents in a

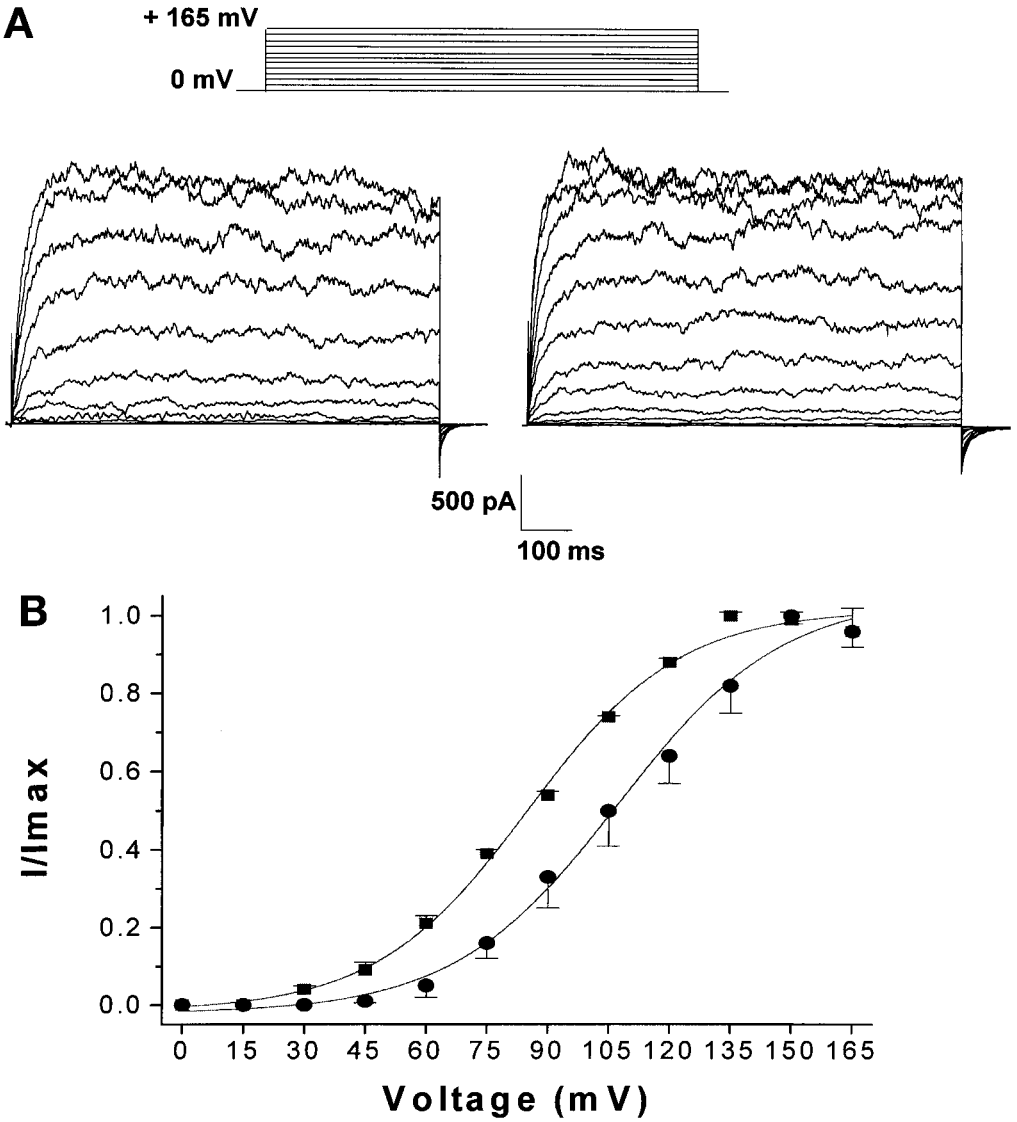


Figure 8. Effect of NH_2Cl on conductance of inside-out patches. Individual patches were stepped from 0 to 165 mV in 15-mV increments for 800 milliseconds to generate steady-state macroscopic currents, before and after application of 6 μmol/L NH_2Cl. (*A*) Current traces are averages of 3 trials. The maximum patch current was not increased after exposure to NH_2Cl. (*B*) Peak patch current was taken as the average current measured during the last 200 milliseconds of the voltage step. Steady-state voltage dependence of activation was determined by normalizing currents against peak current for a given patch and fitting these data to a Boltzmann equation. NH_2Cl shifted the midpoint ($V_{1/2}$) of the Boltzmann relationship without altering peak patch conductance or slope value (see text). This set of experiments was performed using 100 nmol/L Ca^{2+} in the solution bathing the cytosolic face of patches. Data represent mean ± SEM for 4 patches.

dose-dependent manner. These outward currents were shown to reflect a pure K^+ conductance by analysis of tail current reversal potential at varying K^+ gradients. NH_2Cl-enhanced currents were inhibited by extracellular TEA but not 4-aminopyridine or apamin, indicating that the currents arose from activation of maxi K^+ channels. Single channel studies in excised, inside-out patches showed that NH_2Cl increased open probability of maxi K^+ channels (257 pS) and shifted the voltage dependence of steady-state activation of single channels by 22 mV. This shift may be sufficient to increase the open probability of the large-conductance channel near the resting membrane potential of TMM cells, thereby leading to membrane hyperpolarization. This is consistent with our finding that in whole tissue strip recordings, NH_2Cl-induced hyperpolarization was inhibited by IBTX. However, the effects of NH_2Cl on other resting K^+ conductances cannot be entirely ruled out.

Because the membrane-impermeant compound taurine NHCl, an analogue of NH_2Cl, was ineffective in enhancing K^+ currents in whole cell voltage clamp experiments, the mechanism of NH_2Cl action is likely to involve modulation from the cytosolic compartment. This is consistent with previous findings that NH_2Cl can induce DNA damage and can cause detachment of fetal cardiac myocytes in a tyrosine kinase–dependent fashion.[28–30]

Tamai et al.[23] showed that activation of epithelial Cl^- secretion in colonic T84 monolayers by NH_2Cl resulted from an increase in cytosolic Ca^{2+}. In this study of ionic currents in cells from the rabbit colonic TMM, NH_2Cl enhanced outward whole cell currents more than 8-fold at +30 mV in the presence of high intracellular Ca^{2+} buffering, indicating a Ca^{2+}-independent mechanism of action. Our experiments also showed that maxi K^+ channel activation by NH_2Cl occurs in inside-out patches

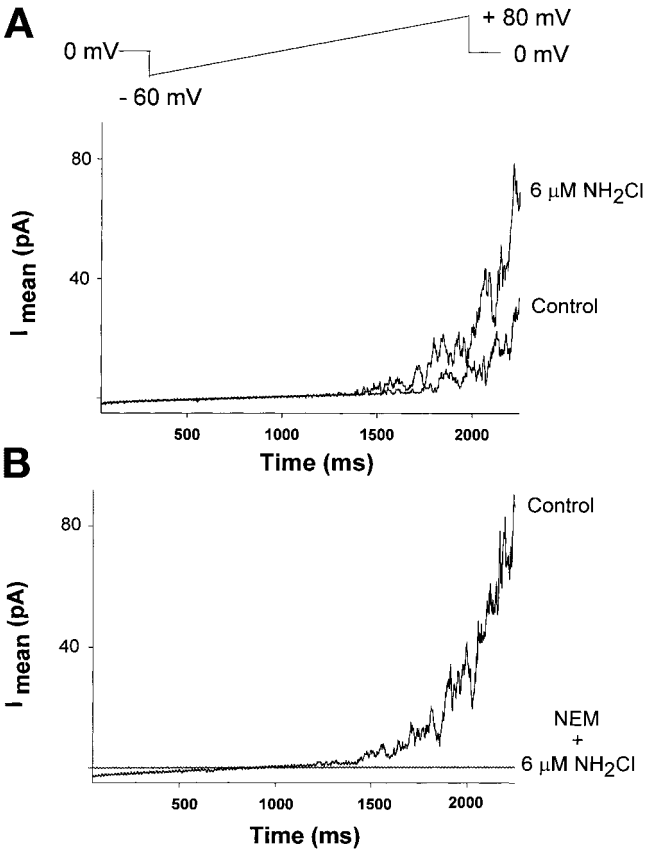


Figure 9. Effect of SH alkylation on NH_2Cl-enhanced current. Inside-out patches were treated with 1 mmol/L NEM before incubation with NH_2Cl. NEM alkylates SH groups, preventing modification by oxidants. Voltage ramps were applied as shown to generate macroscopic patch currents. (*A*) Patch not pretreated with NEM. NH_2Cl increases mean current. (*B*) Patch pretreated with NEM for 45 minutes. Mean current is markedly reduced after NEM treatment. The NH_2Cl-enhanced currents are eliminated after SH alkylation by NEM.

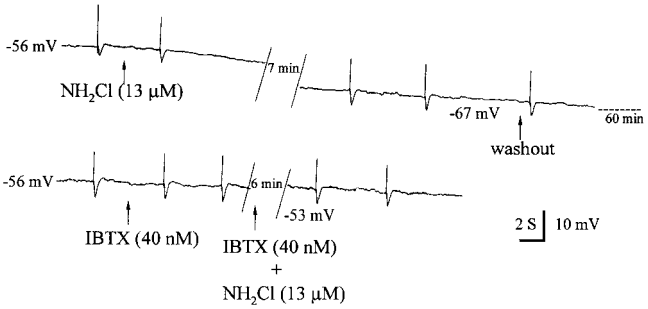


Figure 10. Effect of NH_2Cl on membrane potential in rabbit colonic circular muscle. Upper trace NH_2Cl (13 μmol/L) induced an 11-mV hyperpolarization that is not reversed on washout. *Lower trace*, exposure to IBTX (40 nmol/L) has no effect on membrane potential but prevents NH_2Cl-induced hyperpolarization.

perfused with a Ca^{2+}-free bath solution containing high EGTA, indicating that NH_2Cl exerts a direct effect on the channel protein in a Ca^{2+}-independent fashion. This current was insensitive to modulation by the nonselective tyrosine kinase inhibitor genistein and the tyrosine phosphatase inhibitor orthovanadate (data not shown).

NH_2Cl avidly oxidizes amino acid SH groups.[12] The SH alkylating agent NEM prevented NH_2Cl-induced channel activation. This confirms that the observed increase in maxi K^+ channel activity caused by NH_2Cl results from oxidation of SH residues on the channel protein. Prolonged exposure (1 hour) to NEM completely inhibited channel activity. These data imply that, in colonic TMM smooth muscle, SH group modification represents a tertiary level of maxi K^+ channel regulation that may be of particular importance during conditions of cellular stress that result in an altered intracellular redox state.

The diverse effects of reactive oxygen species on K^+ channels have been reviewed recently.[31] Cai and Sauvé[32] showed that H_2O_2 reversibly inhibited the intermediate-conductance, Ca^{2+}-activated K^+ channel in bovine aortic endothelial cells. Nakaya et al.[33] showed that reduction of whole cell currents by cumene hydroperoxide in guinea pig ventricular myocytes was caused by inhibition of the inwardly rectifying K^+ channel, and that this occurred without alteration of single channel amplitude. Wang et al.[34] showed that the maxi K^+ channel in equine tracheal smooth muscle is subject to modulation by redox reactions. In contrast to Wang et al., who showed that SH oxidation inhibited the activity of the maxi- K^+ channel, our data show activation of the maxi K^+ channel in colonic smooth muscle by the SH oxidizing compound NH_2Cl. This apparent discrepancy may be explained by the work of Park et al.,[35] who have shown that redox modulation of the maxi K^+ channel even within the same organism is a tissue-specific phenomenon. Furthermore, it has been suggested that the physical properties of NH_2Cl enable it to react with SH residues in proteins that are otherwise not amenable to modification by other oxidants.[15]

Being the innermost layer of smooth muscle, the TMM lies adjacent to the lamina propria and may be a major target for inflammatory mediators such as platelet-derived growth factor, leukotriene D_4, and the terminal complement complex.[36–38] Percy et al.[37] have shown that net colonic contractile state may be selectively mediated by the TMM. It is likely that the TMM coordinates movement of mucosal villi, a process underlying the secretory and absorptive functions of the gut.[39] Because mucosal end arteries traverse the TMM, the contractile state of this smooth muscle layer may ultimately regulate mucosal blood flow.[40,41]

In summary, our data show that NH_2Cl, an oxidant of special importance during colitis, may contribute to the pathophysiology found in this condition by a direct

action on the muscle cell membrane. The lipophilic nature of NH_2Cl enables it to easily enter smooth muscle cells and oxidize SH residues on the maxi K^+ channel. Modification of these SH residues leads to a shift in the voltage dependence of channel activation, resulting in enhancement of outward currents accompanied by membrane hyperpolarization at concentrations of NH_2Cl within the range expected to be present during colitis. Decreased electrical excitability of this muscle layer may affect mucosal movement and permit enhanced bacterial adherence, exaggerating chlorinated oxidant production, thereby contributing to smooth muscle dysfunction present during colitis.

References

1. Spriggs EA, Code CF, Bargan JA, Curtiss RK, Hightower NC. Motility of the pelvic colon and rectum of normal persons and patients with ulcerative colitis. Gastroenterology 1951;19:480–491.
2. Snape WJ Jr, Kao HW. Role of inflammatory mediators in colonic smooth muscle function in ulcerative colitis. Dig Dis Sci 1998;33: 65S–70S.
3. Myers BS, Martin JS, Dempsey DT, Parkman HP, Thomas RM, Ryan JP. Acute experimental colitis decreases colonic circular smooth muscle contractility. Am J Physiol 1997;273:G928–G936.
4. Crosthwaite AIP, Huizenga JD, Fox JET. Jejunal circular muscle motility is decreased in nematode-infected rats. Gastroenterology 1990;98:59–65.
5. Jacobson K, McHugh K, Collins SM. Experimental colitis alters myenteric nerve function at inflamed and noninflamed sites in the rat. Gastroenterology 1995;109:718–722.
6. Rao, SSC, Read NW. Gastrointestinal motility in patients with ulcerative colitis. Scand J Gastroenterol Suppl 1990;172:22–28.
7. Koch, TR, Carney JA, Go VLW, Szurszewski JH. Spontaneous contractions and some electrophysiological properties of circular muscle from normal sigmoid colon and ulcerative colitis. Gastroenterology 1988;95:77–84.
8. Snape WJ Jr, Williams R, Hyman PE. Defect in colonic smooth muscle function in ulcerative colitis. Am J Physiol 1991;261:G987–G991.
9. Lu G, Qian X, Berezin I, Telford GL, Huizenga JD, Sarna SK. Inflammation modulates in vitro colonic myoelectric and contractile activity and interstitial cells of Cajal. Am J Physiol 1997;273: G1233–G1245.
10. Rumessen JJ. Ultrastructure of interstitial cells of Cajal at the colonic submuscular border in patients with ulcerative colitis. Gastroenterology 1996;111:1447–1455.
11. Wrong O. The metabolism of ammonia and urea in the healthy and uraemic colon. Med J Aust 1967;2:281–283.
12. Thomas EL, Grisham MB, Jefferson MM. Myeloperoxidase-dependent effect of amines on functions of isolated neutrophils. J Clin Invest 1983;72:441–454.
13. Agner K. Biological effects of hypochlorous acid formed by ''MPO''-peroxidation in the chloride ion. In: Akeson A, Ehrenberg A, eds. Structures and functions of oxidation-reduction enzymes. London: Pergamon, 1972:329–335.
14. Grisham MB, Jefferson MM, Melton DF, Thomas EL. Chlorination of endogenous amines by isolated neutrophils. J Biol Chem 1984;259:10404–10413.
15. Grisham MB, Jefferson MM, Thomas EL. Role of monochloramine in the oxidation of erythrocyte hemoglobin by stimulated neutrophils. J Biol Chem 1984;259:6757–6765.
16. Weiss SJ, Lampert MB, Test ST. Long-lived oxidants generated by human neutrophils: characterization and bioactivity. Science 1983;222:625–628.
17. Weiss SJ, Curnutte JT, Regiani S. Neutrophil mediated solubilization of the subendothelial matrix: oxidative and nonoxidative mechanisms of proteolysis used by normal and chronic granulomatous disease phagocytes. J Immunol 1986;136:636–641.
18. Ossanna PJ, Test ST, Matheson NR, Regiani S, Weiss SJ. Oxidative regulation of neutrophil elastase-alpha-1-proteinase inhibitor interactions. J Clin Invest 1986;77:1939–1951.
19. Shih KL, Lederberg J. Chloramine mutagenesis in *Bacillus subtilis*. Science 1976;192:1141–1143.
20. Buckley JA. Intoxication of trout erythrocytes from monochloramine in vitro oxidative alteration and recovery of hemoglobin and glutathione. Comp Biochem Physiol C 1981;69:337–344.
21. Grisham MB, Gaginella TS, von Ritter C, Tamai H, Be RM, Granger DN. Effects of neutrophil-derived oxidants on intestinal permeability, electrolyte transport, and epithelial cell viability. Inflammation 1990;14:531–542.
22. Tamai H, Kachur JF, Baron DA, Grisham MB, Gaginella TS. Monochloramine, a neutrophil-derived oxidant, stimulates rat colonic secretion. J Pharmacol Exp Ther 1990;257:884–894.
23. Tamai H, Gaginella TS, Kachur JF, Musch MW, Chang EB. Ca-mediated stimulation of Cl secretion by reactive oxygen metabolites in human colonic T84 cells. J Clin Invest 1992;89: 301–307.
24. Hatakeyama N, Mukopadhyay D, Goyal RK, Akbarali HI. Tyrosine kinase–dependent modulation of calcium entry in rabbit colonic muscularis mucosae. Am J Physiol 1996;270:C1780–C1789.
25. Thomas EL, Grisham MB, Jefferson MM. Preparation and characterization of chloramines. Methods Enzymol 1986;132:569–584.
26. Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ. Improved patch clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflugers Arch 1981;391: 85–100.
27. Carl A, Sanders KM. Measurement of single channel open probability with voltage ramps. J Neurosci Methods 1990;33:157–163.
28. Bernofsky C. Nucleotide chloramines and neutrophil-mediated cytotoxicity. FASEB J 1991;5:295–300.
29. Suzuki H, Seto K, Mori M, Suzuki M, Miura S, Ishii H. Monochloramine-induced DNA fragmentation in gastric cell line MKN 45. Am J Physiol 1998;275:G712–G716.
30. Nakamura TY, Yamamoto I, Nishitani H, Matozaki T, Suzuki T, Wakabayashi S, Shigekawa M, Goshima K. Detachment of cultured cells from the substratum induced by the neutrophil-derived oxidant NH_2Cl: synergistic role of phosphotyrosine and intracellular Ca^{2+} concentration. J Cell Biol 1995;131:509–524.
31. Kourie JI. Interaction of reactive oxygen species with ion transport mechanisms. Am J Physiol 1998;275:C1–C24.
32. Cai S, Sauvé R. Effects of thiol-modifying agents on a K(Ca^{2+}) channel of intermediate conductance. J Membr Biol 1997;158: 147–158.
33. Nakaya H, Tohse N, Kanno M. Electrophysiologic derangements induced by lipid peroxidation in cardiac tissue. Am J Physiol 1987;253:H1089–H1097.
34. Wang Z, Nara M, Wang Y, Kotlikoff MI. Redox regulation of large conductance Ca^{2+}-activated K^+ channels in smooth muscle cells. J Gen Physiol 1997;110:35–44.
35. Park MK, Bae YM, Lee SH, Ho WK, Earm YE. Modulation of voltage dependent K^+ channel by redox potential in pulmonary and ear arterial smooth muscle cells of the rabbit. Pflugers Arch 1997;434:764–771.

36. Hu XQ, Singh N, Mukopadhyay D, Akbarali HI. Modulation of voltage-dependent Ca^{2+} channels in rabbit colonic smooth muscle cells by c-Src and focal adhesion kinase. J Biol Chem 1998;273: 5337–5342.
37. Percy WH, Lee SY, Burton MB, Tolentino T, Burakoff R. Leukotriene D4 excitation of rabbit distal colon arises in the region of the muscularis mucosae. Am J Physiol 1990;259:G753–G759.
38. Halstensen TS, Mollnes TE, Fausa O, Brandtzaeg P. Deposits of terminal complement complex (TCC) in muscularis mucosae and submucosal vessels in ulcerative colitis and Crohn's disease of the colon. Gut 1989;30:361–366.
39. King CE, Robinson MH. The nervous mechanisms of the muscularis mucosae. Am J Physiol 1945;143:325–335.
40. Piasecki C, Thrasivoulou C, Rahim A. Ulcers produced by ligation of individual gastric mucosal arteries in the guinea pig. Gastroenterology 1989;97:1121–1129.
41. Piasecki CK, Thrasivoulou C. Spasm of gastric muscularis mucosae might play a key role in causing focal mucosal ischemia and ulceration: an experimental study in guinea pigs. Dig Dis Sci 1993;38:1183–1189.

Received February 5, 1999. Accepted June 22, 1999.

Address requests for reprints to: Hamid I. Akbarali, Ph.D., Research and Development, 151, West Roxbury Veterans Affairs Medical Center, 1400 VFW Parkway, Boston, Massachusetts 02132. e-mail: hakbarali@hms.harvard.edu; fax: (617) 363-5592.

Supported by National Institutes of Health grants DK-46367 (to H.I.A.); DK-48010 and DK-51630 (to J.B.M.); training grant (NIH) GMO-780617 (to M.P.); and the George H.A. Clowes, Jr., M.D., Fellow of the American College of Surgeons Memorial Career Development Award from the American College of Surgeons (to J.B.M.).

The authors thank Dr. Raj K. Goyal for kindly reading the manuscript and for his suggestions and comments.