Differential modulation of voltage-dependent K⁺ currents in colonic smooth muscle by oxidants

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Departments of ¹Surgery and ²Research and Development, Veterans Affairs Medical Center, West Roxbury 02132; and ³Department of Surgery, Brigham and Women’s Hospital, ⁴Harvard Medical School, Boston, Massachusetts 02115

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Prasad, Madhu, and Raj K. Goyal. Differential modulation of voltage-dependent K⁺ currents in colonic smooth muscle by oxidants. Am J Physiol Cell Physiol 286: C671–C682, 2004. First published November 12, 2003; 10.1152/ajpcell.00137.2003.—The effect of oxidants on voltage-dependent K⁺ currents was examined in mouse colonic smooth muscle cells. Exposure to either chloramine-T (Ch-T), an agent known to oxidize both cysteine and methionine residues, or the colon-specific oxidant monochloramine (NH₂Cl) completely suppressed the transient outward K⁺ current (Iₒ) while simultaneously enhancing the sustained delayed rectifier K⁺ current (I_dr). In contrast, the cysteine-specific oxidants hydrogen peroxide (H₂O₂) and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) exhibited partial and slow suppression of Iₒ by inducing a shift in channel availability of −18 mV without affecting I_dr. After enhancement by NH₂Cl or Ch-T, Iₒ was sensitive to 10 nM tetraethylammonium but not to other K⁺ channel blockers, suggesting that it represented activation of the resting Iₒ and not a separate K⁺ conductance. Extracellular dithiothreitol (DTT) partially reversed the effect of H₂O₂ and DTNB on Iₒ, but not the actions of NH₂Cl and Ch-T on either Iₒ or I_dr. Dialysis of myocytes with GSH (5 mM) or DTT (5 mM) prevented suppression of Iₒ by H₂O₂ and DTNB but did not alter the effects of NH₂Cl or Ch-T on either Iₒ or I_dr. Ch-T and NH₂Cl completely blocked Iₒ, generated by murine Kv4.1, 4.2, and 4.3 in Xenopus oocytes, an effect not reversible by intracellular DTT. In contrast, intracellular DTT reversed the effect of H₂O₂ and DTNB on the cloned channels. These results suggest that Iₒ is suppressed via modification of both methionine and cysteine residues, whereas enhancement of I_dr likely results from methionine oxidation alone.

colon; colitis; redox; ion channel

DURING COLITIS, activated polymorphonuclear neutrophils (PMN) in the colonic mucosa and submucosa release reactive oxygen species (ROS) into the bowel wall. ROS such as superoxide (O₂⁻), hydroxyl radical (OH⁻), hypochlorous acid (HOCl), and hydrogen peroxide (H₂O₂) directly mediate many of the injurious effects of inflammation on colonic tissues (4, 28). In addition to their direct effect on cellular function, conventional products of the PMN oxidative burst combine with ammonia (NH₃) present at high levels within the colon (10–70 mM luminal) to produce a group of colon-specific amine-based oxidants such as monochloramine (NH₂Cl).

The effects of ROS on colonic epithelial cells have been widely studied. Oxidants increase junctional permeability, stimulate epithelial Cl⁻ secretion, and thus contribute to the impaired salt and water absorption characteristic of colitis (6, 11). The role of ROS as mediators of smooth muscle dysfunction present during colitis is not well defined, although muscle strips from the colon of humans and animals with colitis exhibit variable changes in contractile activity (15, 22, 30, 38).

Emerging evidence indicates that chemical oxidation and reduction may serve to modulate the activity of ion channels (18, 25, 36, 45). This may be of particular significance in pathological conditions associated with oxidative stress. Colonic myocytes from dogs with chemically induced colitis exhibit reduced Ca²⁺ and K⁺ current density (26, 27). A separate report (1) demonstrates suppression of L-type Ca²⁺ current and concurrent activation of glibenclamide-sensitive K⁺ current in colonic smooth muscle cells from mice with colitis induced by dextran sulfate.

We have previously shown (34) that NH₂Cl enhances activity of large-conductance, Ca²⁺-activated K⁺ channels (BKCa) in rabbit colonic smooth muscle cells. In contrast to this finding, we recently reported (33) that NH₂Cl completely suppresses the transient outward K⁺ channel (Iₒ) in colonic myocytes of the mouse, an effect that is reproduced in cloned homotetramers of the channel α-subunit in Xenopus oocytes. The present series of experiments demonstrates differential modulation of two voltage-dependent K⁺ currents, Iₒ and the delayed rectifier K⁺ current (I_dr), by ROS in single smooth muscle cells of the mouse colon. Like NH₂Cl, the oxidizing agent chloramine-T (Ch-T) abolishes Iₒ while simultaneously enhancing I_dr. Although the cysteine-specific oxidants H₂O₂ and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) partially inhibit Iₒ, they do not affect I_dr. Our results suggest that individual ROS can exert divergent effects on distinct K⁺ channels as a function of time, dose, and site of action.

EXPERIMENTAL PROCEDURES

Preparation of single cells. Mice (C57BL/6) were housed in accordance with guidelines established by the Animal Care Committee at the Veterans Affairs Medical Centers in Boston, Massachusetts, and Portland, Oregon, and also the Oregon Health and Science University. After animals were killed, the abdomen was opened and the distal 2 cm of colon were excised and placed immediately in a Sylgard-coated petri dish containing low-Ca²⁺ Tyrode solution gassed with 95% O₂-5% CO₂. The colon was opened along its mesenteric border; fecal pellets and residue were removed and pinned mucosa side down. Muscle tissue was divided into small fragments and allowed to equilibrate at room temperature (20–25°C) in the gassed solution for 30 min.

Single smooth muscle cells were prepared by enzymatic digestion of the intact tissue. Briefly, the dissected colonic tissue was triturated with a wide-bore Pasteur pipette for 15 min in a cocktail containing collagenase (0.5 mg/ml), trypsin (0.1 mg/ml), and bovine serum albumin.
albumin (1 mg/ml) in low-Ca²⁺ Tyrode solution at room temperature. After dispersal, the solution of cells was stored at 4°C in enzyme-free, low-Ca²⁺ Tyrode solution. For experiments, cells were placed in a perfusion chamber mounted on the stage of an inverted phase-contrast microscope (Carl Zeiss). Individual myocytes were allowed to settle to the bottom of the chamber and then superfused with HEPES-buffered Tyrode solution (pH 7.4) at room temperature. All experiments were carried out within 4 h after cell dispersal.

Oocyte recordings and molecular biology. Xenopus laevis females were obtained from Nasco (Fort Atkinson, WI). No animal underwent more than two operations, with procedures separated by 3 wk or more. Harvested oocytes were dissociated in 1% collagenase A in OR-2 (in mM: 82.5 NaCl, 2 KCl, 1 MgCl₂, 5 HEPES), washed several times and injected with 50 nl of mRNA (20–50 ng), and assayed 2–5 days after injection.

Kᵥ 4.1 (GenBank accession no. NM 008423) was the generous gift of Dr. Lawrence Salkoff (St. Louis, MO); Kᵥ 4.2 (AAD16972) and Kᵥ 4.3 (AAD16973) were generously provided by Dr. Wayne Giles (Calgary, AB, Canada). Constructs were subcloned into the oocyte expression vector pBF. In vitro mRNA was synthesized from pBF constructs, using SP6 polymerase (Invitrogen, Gaithersburg, MD).

Solutions and chemicals. Dissection of smooth muscle and enzymatic dispersal of single cells was carried out in low-Ca²⁺ Tyrode solution containing (in mM) 135 NaCl, 2.7 KCl, 0.33 NaH₂PO₄, 11 NaHCO₃, 1 MgCl₂, 0.01 CaCl₂, and 5.5 glucose. The external solution employed to record K⁺ currents contained (in mM) 135 NaCl, 5.4 KCl, 0.33 NaH₂PO₄, 5 HEPES, 1 MgCl₂, 1.8 CaCl₂, and 5.5 glucose and was adjusted to pH 7.4 with NaOH. Nifedipine (1 µM) was included to prevent Ca²⁺ entry into the cells through L-type Ca²⁺ channels and facilitate isolation of Ca²⁺-independent K⁺ currents. Muscle cells were dialyzed with an internal solution containing (in mM) 100 K-aspartate, 30 KCl, 1.8 CaCl₂, and 5 HEPES, adjusted to pH 7.4 with NaOH. DTT was directly injected into oocytes in some experiments. This was performed by introducing a sharp micropipette into the oocyte and injecting 50 nl of 1 M DTT during the course of a recording (estimated 10 mM final [DTT]). Data were obtained only from those cells in which holding current remained unaffected by this manipulation.

NH₂Cl and taurine monochloramine (tau-NHCl) were synthesized as described previously (34). These compounds as well as DTT, H₂O₂, and DTNB were added to the perfusing solution in the

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Fig. 1. Effect of chloramine-T (Ch-T) and NH₂Cl on voltage-dependent, Ca²⁺-independent K⁺ currents in mouse colonic smooth muscle cells. A: cells held at −80 mV and then stepped to −20 mV for 500 ms exhibited a rapidly activating and inactivating transient outward current (Iₒ) and a sustained component (Iₜₒ). Exposure to 100 µM Ch-T for 5 min completely abolished Iₒ and enhanced Iₜₒ, effects not reversible by simple washout. Even after activation by Ch-T, Iₒ is inhibited by TEA (10 mM). B: family of outward currents obtained in colonic myocyte before and after treatment with Ch-T (100 µM) for 5 min. Cells were held at −80 mV and then stepped in 10-mV increments from −180 mV to −60 mV for 500 ms every 10 s. As with Ch-T, NH₂Cl simultaneously blocked Iₒ while enhancing Iₜₒ. C: current-voltage (IV) plot demonstrates the effect of Ch-T (●) and NH₂Cl (○) on Iₒ and Iₜₒ. Both agents abolished Iₒ and enhanced Iₜₒ, with Iₒ being TEA sensitive before and after exposure to the oxidants. Data are expressed as means ± SE for 28 experiments.
concentrations indicated. Solvent pH was not affected by addition of these compounds in the concentrations indicated.

Electrophysiological methods. Membrane currents were recorded in smooth muscle cells with the use of standard gigaseal patch-clamp techniques in conventional whole cell configuration (19). Pipettes were pulled from borosilicate tubes 1.5 mm in diameter (WPI, Sarasota, FL) with the use of a Flaming/Brown-type puller (P87; Sutter Instruments, Novato, CA) and then fire-polished on a microforge. Pipette resistances were 2–4 MΩ when filled with intracellular recording solution. Currents were recorded with an EPC-9 patch-clamp amplifier (Heka Elektronik, Lambrecht/Pfalz, Germany) controlled by PULSE software (Heka) and were digitized onto the hard drive of an Apple PowerPC desktop computer. As with the stimulus protocols were controlled by PULSE software (Heka) and were digitized onto the hard drive of an Apple PowerPC desktop computer. Data analysis was performed using Igor, and linear leak and capacitance were corrected using a P/4 protocol.

Two-electrode voltage-clamp recordings were made in oocytes. Currents were measured by using a GeneClamp 500 amplifier (Axon Instruments, Foster City, CA), digitized with an ITC-16 data-acquisition interface (InstruTech, Port Washington, NY), and stored on the hard drive of an Apple PowerPC desktop computer. As with the myocyte recordings, stimulus protocols were controlled by PULSE software, data analysis was performed using Igor, and linear leak and capacitance were corrected using a P/4 protocol.

All values are presented as means ± SE. Student’s t-test was used at the 0.05 confidence level to determine the significance between any two means.

Drugs. Ch-T, DTT, GSH, DTNB, H2O2, nifedipine, and catalase were obtained from Sigma (St. Louis, MO). Collagenase A was from Boehringer Mannheim. Collagenase used to prepare single smooth muscle cells was from Yakult Pharmaceutical (Tokyo, Japan). All other chemicals were reagent grade. Stock solutions of DTNB (10 M) and nifedipine (0.1 M) were prepared in DMSO and ethanol, respectively.

Table 1. Biophysical and kinetic parameters of \(I_{\text{so}}\) after exposure of smooth muscle cells to Ch-T

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Ch-T</th>
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<tr>
<td>(t_{1/2}) (activation)</td>
<td></td>
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<tr>
<td>−20 mV</td>
<td>5.4 ± 0.4 ms</td>
<td>5.5 ± 0.4 ms</td>
</tr>
<tr>
<td>+20 mV</td>
<td>3.7 ± 0.3 ms</td>
<td>3.6 ± 0.3 ms</td>
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<tr>
<td>(\tau_{\text{fast}}) (inactivation)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>−20 mV</td>
<td>112 ± 9 ms</td>
<td>117 ± 6 ms</td>
</tr>
<tr>
<td>+20 mV</td>
<td>13 ± 0.8 ms</td>
<td>12 ± 0.8 ms</td>
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<tr>
<td>(\tau_{\text{slow}}) (inactivation)</td>
<td></td>
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<tr>
<td>−20 mV</td>
<td>5,430 ± 240 ms</td>
<td>5,510 ± 260 ms</td>
</tr>
<tr>
<td>+20 mV</td>
<td>5,720 ± 290 ms</td>
<td>5,640 ± 270 ms</td>
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<tr>
<td>(V_{1/2})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>−20 mV</td>
<td>−71 ± 4 mV</td>
<td>−73 ± 3 mV</td>
</tr>
<tr>
<td>+20 mV</td>
<td>81 ± 7 ms</td>
<td>76 ± 9 ms</td>
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The concentration of chloramine T (Ch-T; 10 μM) used to measure these parameters was beneath the threshold for complete channel blockade. Ch-T did not modify apparent kinetics of current activation or the fast (\(\tau_{\text{fast}}\)) or slow (\(\tau_{\text{slow}}\)) time constants of inactivation. The \(V_{1/2}\) of voltage-dependent channel availability was not appreciably altered by Ch-T. The time to half-maximal effect (\(t_{1/2}\)) for recovery from inactivation was not changed after treatment with Ch-T. Data are expressed as means ± SE for 6–8 experiments each. Note that \(\tau_{\text{rate}}\) is not voltage dependent, whereas \(\tau_{\text{rate}}\) decreases at more positive test potentials. \(I_{\text{so}}\), transient outward \(K^+\) current.

Fig. 2. A: dose dependence of \(NH_2Cl\) and Ch-T effects on \(I_{\text{so}}\) and \(I_{\text{DR}}\). Both agents exhibited dose-dependent modulation of \(I_{\text{so}}\) (●) and \(I_{\text{DR}}\) (◇). The effect of \(NH_2Cl\) on \(I_{\text{so}}\) was less potent than its effect on \(I_{\text{DR}}\). Ch-T exhibited similar characteristics. The effect of \(NH_2Cl\) on both \(I_{\text{so}}\) and \(I_{\text{DR}}\) was more potent than corresponding effects of Ch-T (see RESULTS for details). B: effect of Ch-T on \(I_{\text{so}}\) in Xenopus oocyte expressing homotetrameric \(mK_v\) 4.3. Ch-T blocked \(I_{\text{so}}\) generated by the cloned channel, suggesting that it has a direct effect on the pore-forming \(\alpha\)-subunit. Note that outward tail current flattened after exposure to Ch-T, confirming abolition of \(K^+\) current.
tively. Currents were not modified by vehicle alone. None of the compounds utilized in this study changed the pH of the internal or external solutions. NaOCl (200 μM), NH₄Cl (10–20 mM), and taurine (1–5 mM) were found to have no effect on the currents examined in this study.

RESULTS

We (33) and others (24) have previously shown the presence of two distinct voltage-dependent K⁺ currents, Iₒ and Iᵩ, in mouse colonic myocytes under Ca²⁺-buffered conditions. Iₒ

![Diagram](image-url)
activates and inactivates rapidly at subthreshold potentials and is inhibited by external 4-aminopyridine (4-AP; 5 mM), whereas \( I_{\text{dr}} \) activates above \(-10 \) mV, does not inactivate, and is inhibited by tetraethylammonium (TEA; 10 mM). In the present series of experiments, use of nifedipine as a Ca\(^{2+}\) channel blocker in place of the divalent cations Co\(^{2+}\) or Mn\(^{2+}\) resulted in marked acceleration of \( I_{\text{dr}} \) inactivation kinetics, likely due to a surface charge phenomenon known to occur in the presence of divalents (14).

**Effects of Ch-T and NH\(_2\)Cl on voltage-dependent K\(^{+}\) currents.** Whole cell Ca\(^{2+}\)-independent currents were recorded in the presence of Ch-T or NH\(_2\)Cl. We have previously demonstrated that NH\(_2\)Cl completely blocks \( I_{\text{dr}} \) at low micromolar concentrations (33). Although those experiments were performed in the presence of TEA (10 mM) to selectively isolate \( I_{\text{dr}} \), TEA was not included in the present studies.

Exposure of cells to Ch-T, which oxidizes both protein methionine and cysteine residues, resulted in complete block of \( I_{\text{dr}} \) at low micromolar concentrations (33). Although those experiments were performed in the presence of TEA (10 mM) to selectively isolate \( I_{\text{dr}} \), TEA was not included in the present studies.

**Fig. 1.** A: blockade of \( I_{\text{dr}} \) by Ch-T. B: voltage dependence of peak current \( I_{\text{dr}} \). C: time dependence of Ch-T action on both voltage-dependent K\(^{+}\) currents. D: time dependence of NH\(_2\)Cl action on both voltage-dependent K\(^{+}\) currents.}

**Fig. 2.** A: blockade of \( I_{\text{dr}} \) by Ch-T. B: voltage dependence of peak current \( I_{\text{dr}} \). C: time dependence of Ch-T action on both voltage-dependent K\(^{+}\) currents. D: time dependence of NH\(_2\)Cl action on both voltage-dependent K\(^{+}\) currents.
forming channel α-subunit (Fig. 2B; data not shown for mKv4.1 and 4.2).

The reversal potential of tail currents as a function of external [K+] confirmed that the outward current enhanced after treatment with Ch-T and NH2Cl represented a K⁺ conductance (Fig. 3, A and B). Reversal potential of tail currents plotted as a function of external [K⁺] revealed a slope of 60 ± 4 mV (n = 6), which closely approximates that predicted by the Nernst equation for a pure K⁺ conductance (Fig. 3B). It was possible that the enhanced sustained outward K⁺ current following exposure to Ch-T or NH2Cl resulted from activation of a K⁺ conductance distinct from Iₒ. Several K⁺ channel blockers were therefore tested for their effectiveness as inhibitors of this current following oxidant activation. Whereas TEA (10 mM) completely inhibited the oxidant-induced increase in Iₒ, several K⁺ blockers were therefore tested for their effectiveness as inhibitors of this current following oxidant activation. Whereas TEA (10 mM) completely inhibited the oxidant-induced increase in Iₒ, ibetisotoxin (10 nM), amarin (3 μM), 4-AP (5 mM), and dendrotoxin-α (200 nM) had no effect (Fig. 3C). Thus the current enhanced by Ch-T and NH2Cl was indeed the 4-AP-insensitive Iₒ and did not represent activation of a separate K⁺ current. Enhancement of Iₒ by NH2Cl and Ch-T in the presence of 4-AP demonstrated that the activated current did not result from changes in fast inactivation of Iₒ (Fig. 3D).

Tau-NHCl, a cell-impermeant NH2Cl analog, did not enhance Iₒ, indicating that NH2Cl appeared to act from within the cytosol (Fig. 3E). Nifedipine has been shown to block the human heart delayed rectifier (hKv1.5) expressed in HEK-293 cells (46). In contrast to the findings with hKv1.5, Iₒ selectively isolated by blocking Iₒ with 4-AP (5 mM) in mouse colonic smooth muscle cells was not sensitive to nifedipine (Fig. 3F).

Effect of H₂O₂ and DTNB on voltage-dependent K⁺ currents. Ch-T is known to oxidize both methionine and cysteine residues on proteins (35). Although it has been established that NH2Cl oxidizes sulfhydryl groups on cysteine amino acids, its methionine functionality has not been defined (17). To determine whether the reciprocal effects of NH2Cl and Ch-T on Idr and Iₒ were due to oxidation of only cysteine residues, we characterized the effects of the cysteine-specific oxidizing agents H₂O₂ and DTNB on Idr and Iₒ. Cells exposed to H₂O₂ exhibited only partial reduction of Iₒ at concentrations as high as 30 mM (Fig. 4, A and B). This contrasted with complete inhibition of Iₒ observed in the presence of Ch-T and NH2Cl. Higher concentrations of H₂O₂ or DTNB caused breakdown of the seal between patch pipette and cell. Neither H₂O₂ nor DTNB was able to completely suppress Iₒ in any cell tested, with maximum inhibition usually 50–60% (Fig. 4B). This effect was less rapid than the inhibition induced by Ch-T and NH2Cl (Fig. 4C). The t₁/₂ values for inhibition of Iₒ by H₂O₂ and DTNB were 8.5 ± 1.3 and 10.2 ± 2.2 min, respectively, significantly slower than with either NH2Cl or Ch-T (n = 6).

Suppression of Iₒ by H₂O₂ and DTNB was not reversible by simply washout, suggesting that this effect was likely mediated by covalent modification. DTNB and H₂O₂ were equally potent in suppressing Iₒ (Fig. 4D), although they were markedly less effective than either Ch-T or NH2Cl.

In contrast to the effect of NH2Cl or Ch-T, DTNB and H₂O₂ shifted availability of Iₒ (Vₜ⁄₂) to more negative test potentials without altering the slope factor (−68 ± 3 mV for control vs. −85 ± 3 mV for H₂O₂ and −84 ± 2 mV for DTNB, n = 8 each; Fig. 5A). Although channel availability was reduced as a function of voltage, the recovery from inactivation was unchanged after exposure to H₂O₂ or DTNB and did not therefore contribute to suppression of Iₒ (t₁/₂ for recovery = 80 ± 7 ms for control vs. 78 ± 8 ms for 10 mM H₂O₂ and 77 ± 7 ms for 5 mM DTNB, n = 8). Neither H₂O₂ nor DTNB altered kinetics of Iₒ activation or inactivation (Fig. 5B).

DTNB and H₂O₂ suppressed Iₒ generated by homotetramers of mKv4.1, 4.2, and 4.3 in Xenopus oocytes, suggesting an action on the channel α-subunit (Fig. 6, A and B). As in colonic smooth muscle, the effect of these cysteine-selective com-

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**Fig. 5.** Effect of DTNB and H₂O₂ on biophysical and kinetic properties of Iₒ. A: voltage-dependent availability of Iₒ (Vₜ⁄₂) before and after treatment with DTNB (10 mM). A family of currents was generated in myocytes held at −80 mV and stepped for 3 s from −100 mV to +20 mV in 10-mV increments, followed by a second pulse to +20 mV for 500 ms. Data at right have been plotted as a function of test potential and then fit to a Boltzmann function. DTNB (■) induces a shift in Vₜ⁄₂ to more negative potentials by 17 mV. Data are presented as means ± SE for 8 cells. Findings are similar with H₂O₂ (▲). B: treatment with H₂O₂ reduced the amplitude of Iₒ but did not affect kinetics of activation or inactivation as illustrated by the superimposed normalized traces at right.
NH$_2$Cl, H$_2$O$_2$ and DTNB did not affect a Boltzmann function, as in Fig. 5. Data are presented as means ± SE for 8 cells.

The effect of reducing agents on voltage-dependent K$^+$ currents. Inhibition of $I_{lo}$ and enhancement of $I_{dr}$ by oxidants were not reversible by simple washout, suggesting that these changes might be due to covalent chemical transformation. Reducing agents have been reported to reverse oxidant-induced alterations in the properties of several native and cloned ion channels (4, 13, 32, 39). We therefore tested whether the cysteine-specific reducing agent DTT would effectively modify oxidant-induced changes in $I_{lo}$ or $I_{dr}$.

DTT alone (5–10 mM) had no effect on $I_{lo}$ or $I_{dr}$ (Fig. 7A). External DTT did not restore $I_{lo}$ after inhibition by NH$_2$Cl or Ch-T (Fig. 7, A and B). Furthermore, external DTT did not reverse activation of $I_{lo}$ induced by NH$_2$Cl or Ch-T (Fig. 7, A and B). These findings suggested that the action of Ch-T, like NH$_2$Cl, might occur from within the cytosol. It is also possible that these agents might modify DTT-resistant amino acid residues.

In contrast to the results in cells exposed to Ch-T or NH$_2$Cl, extracellular DTT partially restored $I_{lo}$ (by 38%) after blockade by the cysteine-specific agents H$_2$O$_2$ or DTNB (Fig. 8, A and B). In data not shown, external DTT partially reversed the shift in $V_{1/2}$ induced by these agents (−85 ± 3 to −78 ± 2 mV for H$_2$O$_2$ and −84 ± 2 to −78 ± 2 mV for DTNB).

The ability of oxidants to modulate $I_{lo}$ and $I_{dr}$ in cells dialyzed internally with DTT or GSH was examined. Internal DTT (5 mM) and GSH (5 mM) had no independent effect on $I_{lo}$ or $I_{dr}$. Both DTT and GSH prevented inhibition of $I_{lo}$ by H$_2$O$_2$ and DTNB (Fig. 9A). $I_{lo}$ was resistant to suppression by H$_2$O$_2$, but not DTNB, in cells dialyzed with catalase, an enzyme that breaks down H$_2$O$_2$ into H$_2$O and O$_2$ (Fig. 9B). This suggests that suppression of $I_{lo}$ by these cysteine-specific oxidants occurred from within the cytosol and that their effect on $I_{lo}$ likely resulted from cysteine oxidation. In contrast, neither DTT nor GSH attenuated the suppression of $I_{lo}$ caused by Ch-T and NH$_2$Cl, and did they not prevent the activation of $I_{dr}$ by these agents, implying that a cysteine-independent mechanism was likely responsible for these changes (Fig. 9C).

The effect of internal DTT on $I_{lo}$ generated by channel clones resembled the results in muscle cells. Although injection of DTT by itself did not alter currents, introduction of DTT into oocytes reversed attenuation of mK$_v$4.3 current induced by H$_2$O$_2$ (Fig. 10). Although not depicted here, results were the same with mK$_v$4.1 and 4.2. The effect on DTNB-treated cells was similar, indicating that H$_2$O$_2$ and DTNB likely inhibited $I_{lo}$ from the cytosolic aspect. Injection of DTT into oocytes did not reverse inhibition induced by NH$_2$Cl or Ch-T, indicating that these oxidants likely affect DTT-resistant amino acid residues affecting channel function (Fig. 11). The effects of redox agents on $I_{lo}$ and $I_{dr}$ are summarized in Table 2.

**DISCUSSION**

The major findings of the present study are that, in smooth muscle cells of the mouse colon, 1) Ch-T, which oxidizes both methionine and cysteine amino acids, abolishes $I_{lo}$ while simultaneously enhancing $I_{dr}$; 2) NH$_2$Cl exerts similar effects on both of these currents but is more potent in its action; 3) the cysteine-selective oxidants H$_2$O$_2$ and DTNB partially suppress $I_{lo}$ but, in marked contrast to the effect of NH$_2$Cl and Ch-T, do not affect $I_{dr}$; and 4) the cysteine-specific reducing agents DTT
and GSH do not independently alter either $I_{to}$ or $I_{dr}$, and they restore $I_{to}$ after inhibition by $H_2O_2$ or DTNB, but they have no effect on changes in $I_{to}$ or $I_{dr}$ induced by NH$_2$Cl or Ch-T. Taken together, these findings suggest that 1) individual oxidants can exert markedly different effects on the various $K^+$ channels in a single smooth muscle cell and 2) chemically distinct oxidants may modulate specific $K^+$ channels in different ways. Both $I_{to}$ and $I_{dr}$ are known to play important roles in

Fig. 7. Effect of external DTT on oxidant-influenced changes in $I_{to}$ and $I_{dr}$. A: extracellular DTT alone did not affect either current, as shown by representative traces from a single experiment. After DTT was washed out, Ch-T blocked $I_{to}$ and enhanced $I_{dr}$, effects that were not reversed by extracellular DTT. B: DTT alone had no effect on either $I_{to}$ or $I_{dr}$. After washout of DTT, Ch-T enhanced $I_{to}$ and suppressed $I_{dr}$. Subsequent exposure to DTT failed to restore currents to control levels.

Fig. 8. Effect of extracellular DTT on $H_2O_2$-induced changes in $I_{to}$. A: blockade of $I_{to}$ by $H_2O_2$ was partially restored by external DTT, as shown by traces from a single cell. B: time dependence of the effect of DTT on $H_2O_2$-treated cells. Data are presented as means ± SE for 8 cells.
the rhythmic electrical activity of the colon; thus changes in their behavior induced by different oxidants might differentially affect motility in colitis.

Ch-T is a mild oxidizing agent that reacts with the exposed thioether group on methionine residues and cysteine residue sulfhydryl groups at pH 7.0–8.5 (37). Ch-T abolishes fast inactivation of the Na$^+$/H$^+$ current in myelinated neurons and skeletal muscle fibers of the toad while enhancing slow inactivation of cloned Na$^+$/H$^+$ channels (42, 43). Ciorba et al. (10) have demonstrated that Ch-T slows the inactivation of cloned Shaker B/C K$^+$/H$^+$ channels expressed in Xenopus oocytes, thereby enhancing $I_{to}$, and that this effect results from oxidation of both methionine and cysteine residues. Ch-T has been found to partially suppress $I_{to}$ and enhance $I_{dr}$ in rabbit atrial myocytes (40). The results presented here differ from these findings in several important aspects. First, $I_{to}$ in colonic smooth muscle cells is completely, and not partially, blocked by Ch-T and NH$_2$Cl. Second, external DTT and internal DTT (or GSH) do not prevent or reverse the effect of Ch-T or NH$_2$Cl on $I_{to}$, suggesting that their effect is not mediated by oxidation of cysteine amino acids. Third, Ch-T activates a glibenclamide-sensitive $I_{dr}$ in rabbit atrial myocytes, whereas $I_{dr}$ in mouse

Fig. 9. Effect of intracellular reducing agents on oxidant-induced modulation of $I_{to}$ and $I_{dr}$. A: dialysis of myocytes with GSH (5 mM) prevented suppression of $I_{to}$ by H$_2$O$_2$ (left). Similarly, cells dialyzed with DTT (5 mM) were resistant to suppression of $I_{to}$ by 5 mM DTNB (right). Internal DTT prevented DTNB-induced inhibition of $I_{to}$ and internal GSH blocked suppression of $I_{to}$ by H$_2$O$_2$ in data not depicted here. B: H$_2$O$_2$ did not suppress $I_{to}$ in smooth muscle cells dialyzed with catalase (200 U/ml); however, the current remained susceptible to DTNB. Catalase specifically detoxified H$_2$O$_2$ but not DTNB. This finding demonstrates that the effect of H$_2$O$_2$ on $I_{to}$ is mediated from within the cytosol. C: intracellular GSH (5 mM) did not alter the effect of Ch-T on either $I_{to}$ or $I_{dr}$ (left). Similarly, intracellular DTT (5 mM) did not prevent NH$_2$Cl-induced inhibition of $I_{to}$ or enhancement of $I_{dr}$ (right). Although relevant data are not depicted here, GSH did not affect the actions of NH$_2$Cl, and DTT did not alter the effects of Ch-T, on either $I_{to}$ or $I_{dr}$. These findings suggest that the actions of Ch-T and NH$_2$Cl on $I_{to}$ and $I_{dr}$ are likely mediated by residues not amenable to cysteine reduction.

Fig. 10. Effect of cysteine-reactive agents on mKv4.3. Oocytes expressing mKv4.3 were held at −80 mV and then stepped to +40 mV for 100 ms every 60 s. A: H$_2$O$_2$ partially suppressed current generated by homotetrameric channel (compare a and b). Current was almost completely restored after oocytes were injected with DTT (c). B: time course of H$_2$O$_2$ and DTT effects. a, b, and c are representative traces from a single cell, reflecting time points as indicated. Extracellular DTT did not reverse H$_2$O$_2$-induced suppression of current. Injection of DTT did not affect currents.
colonic smooth muscle cells is TEA sensitive but glibenclamide insensitive. Finally, apparent activation of \( I_{\text{dr}} \) at low concentration of Ch-T in atrial muscle, unlike colonic muscle, is sensitive to 4-AP and therefore represents inhibition of fast inactivation of \( I_{\text{o}} \). It has been suggested that some of the actions of Ch-T are mediated by the spontaneous production of HOCl in aqueous systems (28). It is unlikely that HOCl mediates the findings in the present study because this compound had no effect on either \( I_{\text{o}} \) or \( I_{\text{dr}} \) in control experiments.

\( \text{NH}_{2}\text{Cl} \) and Ch-T are similar in their time course of action on \( I_{\text{o}} \) and \( I_{\text{dr}} \), and although not addressed in this study, potency differences between these agents may be related to differences in permeability or chemical reactivity. The data presented here show that Ch-T, like \( \text{NH}_{2}\text{Cl} \), has no effect on activation kinetics, inactivation kinetics, voltage-dependent channel availability, or recovery from inactivation. Moreover, Ch-T completely blocks currents generated by homotetramers of mKv4.1, 4.2, and 4.3 in \( \text{Xenopus} \) oocytes, which suggests that its effect on \( I_{\text{o}} \) in smooth muscle cells may be mediated by oxidation of amino acids on the pore-forming channel \( \alpha \)-subunit. Koh et al. (24) have shown that \( I_{\text{o}} \) in murine colonic myocytes results specifically from expression of the \( \text{shal} \) family transcripts encoding mKv4.1, 4.2, and 4.3.

Several novel auxiliary proteins influence the activity of \( I_{\text{o}} \). K\(^{+}\) channel interacting proteins (KChips), K\(^{+}\) channel activating factor, and K\(^{+}\) channel inactivation suppressor modulate channel kinetics but not current magnitude (3, 20, 31). KChips do enlarge the magnitude of \( I_{\text{o}} \) when coexpressed with K\(_{v}4.1\), 4.2, or 4.3; however, this results from increased surface expression. Although an effect of \( \text{NH}_{2}\text{Cl} \) and Ch-T on KChip activity cannot be ruled out, it is unlikely that KChip-induced alteration in surface expression of channels would be responsible for the complete blockade of \( I_{\text{o}} \) observed during the time frame of the experiments presented here.

Ca\(^{2+}\) is known to indirectly influence \( I_{\text{o}} \) inactivation via modulation of calmodulin kinase II (CaMK-II) and protein phosphatase 1 (PP-1) (2, 23). It is doubtful that inhibition of \( I_{\text{o}} \) by any of the ROS used in this study result secondarily from changes in CaMK-II or PP-1 because inactivation of the current is not altered by treatment with oxidants. In addition, \( I_{\text{o}} \) has been measured under stringent low-Ca\(^{2+}\) conditions in the present series of experiments. Complete blockade of \( I_{\text{o}} \) by \( \text{NH}_{2}\text{Cl} \) and Ch-T resembles the effect of the K\(^{+}\) channel blocker 4-AP. None of the auxiliary proteins modulating \( I_{\text{o}} \) activity induces a similar suppression.

Nifedipine has been shown to block the human heart delayed rectifier current (hK\(_{v}1.5\)) expressed in HEK-293 cells (46), and it is therefore possible that enhancement of \( I_{\text{dr}} \) after exposure to Ch-T or \( \text{NH}_{2}\text{Cl} \) results from relief of channel blockade by nifedipine induced by these agents. This is not the likely basis for oxidant enhancement of \( I_{\text{dr}} \) in mouse colonic myocytes because the current was not sensitive to nifedipine in these cells.

### Table 2. Summary of effects of ROS on voltage-dependent currents in mouse colonic smooth muscle cells

<table>
<thead>
<tr>
<th>Current</th>
<th>( \text{Ch-T} )</th>
<th>( \text{NH}_{2}\text{Cl} )</th>
<th>( \text{H}<em>{2}\text{O}</em>{2} )</th>
<th>DTTB</th>
<th>( \text{H}<em>{2}\text{O}</em>{2}/\text{DTNB} )</th>
<th>( \text{TEA} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( I_{\text{o}} )</td>
<td>Complete blockade (100%)</td>
<td>Complete blockade (100%)</td>
<td>Partial blockade (~50%)</td>
<td>Partial blockade (~50%)</td>
<td>Partial reversal of ( \text{H}<em>{2}\text{O}</em>{2}/\text{DTNB} ) blockade</td>
<td>No effect</td>
</tr>
<tr>
<td>( I_{\text{dr}} )</td>
<td>Enhanced</td>
<td>Enhanced</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
<td>Blocked</td>
</tr>
</tbody>
</table>

DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); TEA, tetraethylammonium; \( I_{\text{dr}} \), delayed rectifier K\(^{+}\) current.
The cysteine-selective oxidizing agents H$_2$O$_2$ and DTNB also inhibit $I_{\text{to}}$. Suppression of $I_{\text{to}}$ by H$_2$O$_2$ and DTNB requires concentrations exceeding 3 mM, occurs four- to fivefold more slowly than with Ch-T or NH$_2$Cl, and causes only partial blockade of the current. Although the precise concentration of H$_2$O$_2$ produced by PMNs in vivo is not known, it has been estimated that local [H$_2$O$_2$] can exceed 1–2 mM, and thus suppression of $I_{\text{to}}$ by H$_2$O$_2$ observed in this study may occur during acute inflammation (9). Intracellular dialysis of smooth muscle cells with cysteine-specific reducing agents prevents the action of DTNB and H$_2$O$_2$, suggesting that these compounds exert their effects on $I_{\text{to}}$ from within the cytosol. Oxidation of cysteine residues by H$_2$O$_2$ and DTNB also suppresses currents produced by mK$_v$4.1, 4.2, and 4.3 in Xenopus oocytes, suggesting an effect on the channel α-subunit. Treatment with H$_2$O$_2$ and DTNB shift $V_{1/2}$ of the native current in smooth muscle cells as well as the cloned channel in oocytes to more negative potentials (by 15–17 mV). A recent report (29) demonstrated that treatment of hippocampal neurons with H$_2$O$_2$ produces a marked shift in voltage-dependent availability of $I_{\text{to}}$ by −15 mV, with concurrent suppression of $I_{\text{dr}}$. Inhibition of $I_{\text{to}}$ by arachidonic acid in hippocampal neurons was prevented by intracellular dialysis with cysteine-specific reducing agents, closely resembling the effect in mouse colonic smooth muscle (7).

Unlike Ch-T or NH$_2$Cl, neither DTNB nor H$_2$O$_2$ alters activity of $I_{\text{dr}}$, illustrating another fundamental difference between the two classes of oxidants tested here. It is possible that concentrations of DTNB or H$_2$O$_2$ exceeding those employed in this study might modulate $I_{\text{dr}}$; however, breakdown of giga-seals at higher concentrations (>30 mM) prevented such analysis.

Ch-T enhances the BK$_{Ca}$ channel by oxidation of methionine residues (41). In contrast, oxidation of cysteine residues suppresses both native and cloned BK$_{Ca}$ (12, 44). In a previous report (34), we showed that NH$_2$Cl enhances BK$_{Ca}$ in rabbit colonic smooth muscle. Taken together, these data suggest that Ch-T and NH$_2$Cl modulate at least three separate K$^+$ currents in colonic smooth muscle cells in the same manner: $I_{\text{to}}$, $I_{\text{dr}}$, and BK$_{Ca}$. These effects appear unrelated to cysteine oxidation. NH$_2$Cl mimics the actions of Ch-T and behaves differently from cysteine-specific agents. Although the actions of NH$_2$Cl were not specifically examined in this study, it is possible that, like Ch-T, NH$_2$Cl oxidizes methionine as well as cysteine residues.

In addition to ubiquitous ROS such as H$_2$O$_2$ and HOCl, the high concentration of NH$_3$ in the colon lends itself to the generation of unique amine-based oxidants such as NH$_2$Cl (16). The data presented here suggest that colon-specific oxidants may exert distinct effects on smooth muscle ion channels.

Several reports have indicated that contractile force generation in the colonic wall is altered in colitis (8, 21, 30). Neurons and interstitial cells of Cajal, which both indirectly affect contractile force by their influence on smooth muscle cells, may also be affected by ROS produced during colitis. The results presented here demonstrate that ROS produced in colitis exert a direct effect on the smooth muscle membrane by modulating K$^+$ channels that underlie rhythmic electrical activity. Alteration in the electrical activity of smooth muscle by ROS produced in colitis may play a major role in directly attenuating contractile force as seen in colitis.

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