Paradoxical Effects of Hydrogen Peroxide on Human Airway Anion Secretion

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ABSTRACT

The present study concerns intriguing effects of hydrogen peroxide (H$_2$O$_2$) on cAMP-mediated anion secretion in polarized human airway epithelia. Although H$_2$O$_2$, applied to the apical and basolateral membrane increases short-circuit currents (I$_{sc}$) with analogous properties, it has opposite effects on subsequent CAMP-activated I$_{sc}$ responses. Namely, forskolin (FK)-induced I$_{sc}$ responses were down-regulated by the apical presence of H$_2$O$_2$, whereas they were up-regulated by its basolateral presence. Despite this contrasting effect, oxidative stimuli from either aspect of the monolayer hindered FK-induced increments in cytosolic cAMP levels and apical membrane Cl$^-$ conductance. The site-dependent effects of H$_2$O$_2$ were reproduced in the responses to 8-bromo-cAMP. Estimation of the anionic composition of the I$_{sc}$ revealed that the FK up-regulated both bumetanide [an Na$^+$-K$^+$-2Cl$^-$ cotransporter (NKCC1) inhibitor]-sensitive and 4,4$'$-dinitrostilbene-2,2$'$-disulfonic acid [an HCO$_3^-$-dependent anion transporter (NBC1/AE2) inhibitor]-sensitive I$_{sc}$ in the control, whereas the up-regulation evidently favored bumetanide-sensitive I$_{sc}$ in the basolateral presence of H$_2$O$_2$. The FK-induced NKCC1 augmentation after exposure to basolateral H$_2$O$_2$ was counteracted by cytochalasin D, an inhibitor of microfilament function, but not by charybdotoxin, a blocker of the intermediate conductance Ca$^{2+}$-activated K$^+$ channel, whose activation could be related to NKCC1-mediated Cl$^-$ secretion. These observations suggest that basolaterally but not apically applied H$_2$O$_2$ potentiates subsequent cAMP-mediated Cl$^-$ secretion by an increase in Cl$^-$ uptake via basolateral NKCC1, whose sensitivities to cAMP/protein kinase A are up-regulated, overcoming the H$_2$O$_2$-induced inhibition of cAMP-mediated apical anion conductance. The basolateral membrane-specific effects of H$_2$O$_2$ may be relevant to the basolateral cytoskeleton, which is believed to interact with NKCC1.

Morphologic and physiologic alterations in the human organism are often associated with increases in production of reactive oxygen species (ROS), such as peroxynitrite (ONOO$^-$) and hydrogen peroxide (H$_2$O$_2$) (Bebok et al., 2002). It is well known that ROS damage tissue via direct oxidation of protein, DNA, or lipids (Okayama, 2005). Because of the toxicological effects of ROS, their production by inflammatory cells during episodes of infection and inflammation is responsible for the pathogenesis of a number of respiratory diseases, including bronchial asthma, cystic fibrosis, and chronic obstructive pulmonary disease (Ricciardolo et al., 2006). These ROS-related airway diseases share aspects of mucous congestive diseases (Kellerman, 2002), in which excessive and tenacious mucus secretion causes airway obstruction, and the resultant dysfunction of mucociliary clearance is involved in the morbidity and mortality of these diseases (Rogers, 2005). In vivo, bronchial gland cells contribute to maintenance of effective mucociliary clearance by regulating salt and water secretion via the vectorial ion transport system, thereby forming low-viscosity mucus (Shimura, 2000). Thus, the relationship between ROS and airway ion transport is of considerable interest. The purpose of this work is to elucidate this point, using polarized Calu-3 cells, which may be a model of human airway submucosal gland serous cells (Shen et al., 1994). This cell line expresses high levels of the cystic fibrosis transmembrane conductance regulator (CFTR), a representative anion exit pathway, on
the apical membrane (Haws et al., 1994) and several anion uptake transporters on the basolateral membrane (Loffing et al., 2000). In the present study, we examined the effects of oxidant stress caused by H$_2$O$_2$ on cAMP-dependent anion secretion in polarized human airway serous cell epithelia and found the paradoxical phenomena that the oxidative stimuli applied from the apical or basolateral membrane had opposite effects on cAMP-activated anion secretion.

### Materials and Methods

#### Cell Culture.
Calu-3 human airway cells (American Type Culture Collection, Manassas, VA) at passages 29 through 35 were grown in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Invitrogen), 100 µg/mL streptomycin, and 100 U/mL penicillin (Invitrogen). The cells were maintained in tissue-culture flasks (T75) at 37°C in a humidified 95% air/5% CO$_2$ incubator. After reaching 80 to 90% confluence, cells were detached using a solution of phosphate-buffered saline, 0.04% EDTA, and 0.25% trypsin. The collected cells were passaged with a 1:4 dilution of the same solution and seeded onto porous polyester membranes (0.4-µm pore size on Snapwell or Transwell inserts, 1 cm$^2$; Costar, Cambridge, MA) at a density of 10$^6$ cells/well. The inserts had been coated overnight with 0.2% human placental collagen type VI (Sigma-Aldrich, St. Louis, MO). The day after seeding the cells on the filters, the medium remaining on the apical side was removed to establish an air interface, which markedly improves the differentiation of human airway epithelia in a well polarized fashion (Shen et al., 1994). The cells were fed by replacement of the basolateral medium every 48 h. The cells seeded on the filters normally reached complete confluence, which was confirmed by microscopic observations, in 7 days. Over 13 days, the surface of the monolayers on the filter became clouded day by day. Thus, we determined to carry out our experiments after 7 to 13 days in culture.

#### Bioelectric Studies.
Snapwell inserts on which Calu-3 cells had grown confluent were rinsed with physiological saline solution (PSS) and transferred to modified Ussing chambers (EasyMount Chamber, Physiologic Instruments, San Diego, CA) that contained PSS at 37°C. The PSS was composed of 115 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 2 mM CaCl$_2$, 10 mM glucose, 10 mM Hepes, and 25 mM NaHCO$_3$. The pH of the solution was adjusted to 7.4 (at 37°C) using NaOH before the addition of NaHCO$_3$. The pH of the solution was maintained at 7.4 when gassed with a mixture of 5% CO$_2$ and 95% O$_2$. The monolayers were continuously measured under a short-circuited condition. The ISC value represents the equation of Ohm’s law (Rt = ISC/V). The basolateral solution, CaCl$_2$ was increased to 4 mM to compensate for the Ca$^{2+}$-chelating capacity of the gluconate (Devor et al., 1999).

#### cAMP Assay.
Confluent Calu-3 cells on the permeable supports were exposed to forskolin (FK) (10 µM) for 15 min in the presence of H$_2$O$_2$ and its absence using a cAMP Biotrak enzyme immunoassay kit (Amersham, Arlington, IL). The concentrations of cAMP (1cAMP) in the samples were determined, according to the manufacturer’s instructions. The cAMP levels were expressed as femtomole/well.

#### Chemicals.
FK, 8-bromo-cAMP (8-Br-cAMP), DNDS, NPPB, bumetanide, indomethacin, nystatin, pyruvate, and cytochalasin D (Cyto-D) were obtained from Sigma-Aldrich Co. NS-389 and SC-560 were purchased from Cayman Chemicals (Ann Arbor, MI). H$_2$O$_2$ and charybotoxin (ChTx) were products of Wako Chemical (Tokyo, Japan) and Peptide Institute Inc. (Osaka, Japan), respectively. Stock solutions of 8-Br-cAMP, DNDS, pyruvate, and ChTx were prepared by dissolving them in distilled water. All of the other drugs were dissolved in dimethyl sulfoxide. Nystatin stock solution (100 mM) was made and sonicated for 30 s just before use.

### Results

#### Bioelectric Responses to Apically and Basolaterally Applied H$_2$O$_2$.
The basal $I_{SC}$ and $R_t$ in our experiments using Calu-3 cells were 12.6 ± 0.4 µA/cm$^2$ and 474.4 ± 16.7 Ωcm$^2$, respectively ($n$ = 181). Previous studies have reported that relatively high concentrations of H$_2$O$_2$ ranging from 0.1 to 5 mM, were required to induce barrier dysfunction and anion secretion because airway epithelial cells, including Calu-3, have a strong antioxidant defense capacity (Waters et al., 1997; Zhao and Davis, 1998; Cowley and Linsdell, 2002). As shown in Fig. 1, A and B, the $R_t$ of cells decreased with clearly defined nadirs immediately after exposure to oxidative stimulation of H$_2$O$_2$ (5 mM) from either the apical (A) or basolateral (B) aspect of the membrane, and this parameter gradually returned to its basal level. In a polarized epithelial monolayer, an irreversible increase in monolayer conductance is suggestive of cell damage and loss of viability (Alvarez et al., 1998; Ito et al., 2001). The reversible changes in monolayer $R_t$ observed in the present study indicate that the cells are tolerant of H$_2$O$_2$ at this concentration. Concomitant with the $R_t$ changes, the corresponding peak values in $I_{SC}$ were 73.0 ± 3.6 ($n$ = 5, to apical H$_2$O$_2$) and 49.0 ± 3.3 µA/cm$^2$ ($n$ = 9, to basolateral H$_2$O$_2$), respectively ($p < 0.01$) (Fig. 1, C and D). These bioelectric changes seem likely to include predominantly anion transport because both of them were markedly reduced to 24.0 ± 1.3 ($n$ = 4, $p < 0.01$) and 22.8 ± 2.3 µA/cm$^2$ ($n$ = 4, $p < 0.01$) by the presence of NPPB (100 µM), a Cl$^-$ channel blocker. Previous investigations have shown that airway epithelial cells release cyclooxygenase (COX) products in response to ROS-related stimuli (Matyas et al., 2002). Indomethacin, a COX inhibitor, is generally believed to suppress endogenous production of prostaglandins and thus intracellular cAMP (Mall et al., 1998). As shown in Fig. 1, E through G, the H$_2$O$_2$-induced effects were diminished to 27.2 ± 2.2 ($n$ = 4, $p < 0.01$) and 21.7 ± 1.2 µA/cm$^2$ ($n$ = 4, $p < 0.01$) by pretreatment with indomethacin (10 µM). Similar results were obtained when we used SC-560 (1 µM, a COX-1 inhibitor) and NS-398 (10 µM, a COX-2 inhibitor), resulting in a suppressed peak $I_{SC}$ in response to...
Effects of H$_2$O$_2$ on Intracellular cAMP Production. Based on the data in Fig. 2, we naturally assumed that the IS$_{SC}$ responses to FK would be correlated with the changes in [cAMP]. However, this was not so. As shown in Fig. 3, FK (10 μM)-induced [cAMP] elevation, which had increased from 423.9 ± 61.5 (n = 14, the control) to 14,464.3 ± 3153.1 fmol/well (n = 14, p < 0.01, compared with the control) 15 min after its application, was inhibited by the apical presence of H$_2$O$_2$ (5 mM, 5128.2 ± 1840.0 fmol/well (n = 8, p < 0.01) compared with FK group without H$_2$O$_2$). However, we here observed the paradoxical situation that the FK-induced [cAMP] elevation was also hindered by the oxidative stimuli from the basolateral side (3926.1 ± 826.1 fmol/well (n = 8, p < 0.05) compared with FK group without H$_2$O$_2$), inconsistent with the movement of IS$_{SC}$ (see Fig. 2C). Neither inhibitory effect of H$_2$O$_2$ seems likely to be caused by leakage of
enzymatic reaction with H$_2$O$_2$, which produces acetate, CO$_2$, and restoration of the balance between reduced and oxidized glutathione (Leon et al., 2004). Figure 4, D and E, shows that preincubation with pyruvate (5 mM) counteracted the inhibition of I$_{Cl}$ development as a result of the apical [ΔI$_{Cl}$ = 55.4 ± 2.8 μA/cm$^2$ (n = 4, p < 0.01)] compared with the values in the absence of pyruvate] and basolateral presence of H$_2$O$_2$ [ΔI$_{Cl}$ = 61.4 ± 4.7 μA/cm$^2$ (n = 4, p < 0.01)] compared with the values in the absence of pyruvate]. In addition, acute I$_{Cl}$ changes caused by apical and basolateral H$_2$O$_2$ seem to be up-regulated by the presence of pyruvate.

8-Br-cAMP-Induced Responses in the Presence of H$_2$O$_2$. The aspect-specific effects of H$_2$O$_2$ were reproduced in I$_{Sc}$ responses to the cell-permeable cAMP analog 8-Br-cAMP (1 mM) (Fig. 5, A–C). The I$_{Sc}$ responses to 8-Br-cAMP (58.6 ± 4.4 μA/cm$^2$, n = 12) (Fig. 5A) were down-regulated to 40.8 ± 1.5 μA/cm$^2$ (n = 7, p < 0.01) (Fig. 5B) and up-regulated to 84.9 ± 3.6 μA/cm$^2$ (n = 12, p < 0.01) (Fig. 5C) by apical and basolateral pretreatment, respectively, with H$_2$O$_2$. These observations suggest that cAMP production via adenylyl cyclase is not necessarily a primary target of H$_2$O$_2$ in the innate responses. Similar to the results in Fig. 4, 8-Br-cAMP-augmented I$_{Cl}$ (ΔI$_{Cl}$ = 48.5 ± 1.9 μA/cm$^2$, n = 6) (Fig. 5D) was prevented by preincubation with H$_2$O$_2$ from either the apical (ΔI$_{Cl}$ = 10.1 ± 1.4 μA/cm$^2$, n = 4, p < 0.01) (Fig. 5E) or basolateral surface (21.9 ± 3.0 μA/cm$^2$, n = 4, p < 0.01) (Fig. 5F).

Effects of Basolateral H$_2$O$_2$ on Basolateral Anion Transporters. Based on the apparently paradoxical effects of basolateral H$_2$O$_2$ on the cAMP-mediated I$_{Sc}$, we suspected that up-regulation of the basolateral anion entry rate would
Fig. 7A. Although exposure to basolateral H$_2$O$_2$ further po-
sitivity of ChTx to cAMP-mediated anion secretion (Ito et al.,
1999; Ito et al., 2004a,b). NKCC1 is also activated by
cAMP/protein kinase A (PKA)-mediated phosphorylation
induced NKCC1-Mediated Anion Transport under Baso-
lateral H$_2$O$_2$. The airway epithelial cells secrete Cl$^-$ via
NKCC1 in response to activation of human intermediate
conductance Ca$^{2+}$-activated K$^+$ channels (KCNN4), so that
anion secretion induced by 1-ethyl-2-benzimidazolinone (a
KCNN4 activator) and thapsigargin (a cytosolic Ca$^{2+}$-
mobilizing agent) is markedly inhibited by either bumetanide, a
bikunin (DNDS)sensitive component in FK-in-
activated ISC in the presence and absence of H$_2$O$_2$. After cells reached a
sustained state, bumetanide (Bume) and DNDS were sequentially
applied to estimate the blocker-sensitive components (A). In the basolateral
presence of H$_2$O$_2$, the Bume-sensitive component was selectively aug-
mented (B). Summarized data for the Bume-sensitive and DNDS-sensi-
tive ISC (C and D) are mean ± S.E.M. (n = 7–11). † (p < 0.05) and ‡ (p < 0.01: significantly different from the control
values. * † (p < 0.01) and ‡ (p < 0.05), significant increases and decreases,
respectively, compared with the values in each FK group without H$_2$O$_2$
stress.

Mechanisms Underlying Up-Regulated cAMP-Medi-
linked to the cortical cytoskeleton adjacent to the basolateral
membrane (Matthews, 2002). Thus, we hypothesized that the
cytoskeletal remodeling induced by oxidative stress around
the basolateral membrane could be attributed to the augmenta-
tion of cAMP/PKA-dependent NKCC1 activity. To test this
hypothesis, the effects of H$_2$O$_2$ on FK-induced responses
were observed in the presence of Cyto-D (10 μM), which is
conventionally used to disrupt microfilament function (Mat-
thevs et al., 1997). After the addition of Cyto-D, we observed
gradient and permeabi-
nyltrimethylammonium (nystatin)
the basolateral presence of H$_2$O$_2$ (5 mM), which inhibited
DNDS-sensitive ISC (7.0 ± 2.6 μA/cm$^2$, n = 8) (Fig. 6B). The
data of these observations are summarized in Fig. 6, C and D.

exceed down-regulation of the apical anion export rate. In
airway epithelial cells, including Calu-3 cells, basolateral
anion entry is regulated by several anion transporters, in-
cluding NKCC1 (the bumetanide-sensitive Na$^+$/K$^+$/2Cl$^-$
cotransporter) (Liedtke et al., 2002), NBC1 (the DNDS-sensi-
tive Na$^+$/2HCO$_3^-$ cotransporter) (Inglis et al., 2002), and AE2
(the DNDS-sensitive HCO$_3^-$/Cl$^-$ exchanger) (Loffing et al.,
2000; Inglis et al., 2002). Indeed, FK application similarly
increased both bumetanide- and DNDS-sensitive IS$_{sc}$ from
0.7 ± 0.1 (n = 7) to 11.2 ± 0.8 μA/cm$^2$ (n = 11) and from 0.5 ±
0.1 (n = 7) to 9.9 ± 0.9 μA/cm$^2$ (n = 11), respectively, without
H$_2$O$_2$ (Fig. 6A), whereas the cAMP-mediated up-regulation
evidently favored bumetanide-sensitive IS$_{sc}$ (34.1 ± 2.5 μA/
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with the values in the FK groups pretreated with and without H2O2, H2O2-induced CFTR-mediated anion secretion seems likely increased by exposure to basolateral H2O2 (15.1 cm², Cl⁻). Cyto-D rather offsets the bumetanide-sensitive component in ICl that almost reflect augmentation of CFTR-mediated secretion. This response seems to involve activation of apically located CFTR Cl⁻ channels because of concomitant increases in ICl that almost reflect augmentation of CFTR-mediated anion secretion. Many lines of evidence have shown that the effects of H2O2 on airway muscle contractility are modulated by COX inhibitors or epithelial removal, suggesting the presence of mechanisms to release prostaglandins from airway muscle. The presence of these parameters were inhibited by basolateral H2O2, 60% of maximum conductance at rest, so that the Calu-3 cell is fully capable of anion secretion even under the CFTR-mediated Cl⁻ conductance. Many lines of evidence have shown that the effects of H2O2 on airway muscle contractility are modulated by COX inhibitors or epithelial removal, suggesting the presence of mechanisms to release prostaglandins from airway muscle in response to ROS (Matyas et al., 2002). As shown in the present study, the marked inhibition of apical or basolateral H2O2-induced ICl by indomethacin suggests that H2O2-induced CFTR-mediated anion secretion seems likely to be, at least in part, mediated by intrinsic prostaglandins depending on COX activity. Furthermore, in the responses, both subtypes of COX seem likely to be involved because ISC responses to H2O2 application were equally suppressed by a COX-2 inhibitor (NS-398, Kᵢ of COX-I/COX-2 = 75/1.77 μM) (Barnett et al., 1994) and a COX-1 inhibitor (SC-560, Kᵢ of COX-I/COX-2 = 9 nM/6.5 μM) (Smith et al., 1998). Although the responses to apical and basolateral H2O2 seem likely to be commonly relevant to COX signals, we found that the ISC responses to apical H2O2 were larger than those to basolateral H2O2. These observations allow us to speculate that polarization of the cells produces laterality between the apical and basolateral membrane in the membrane-located COX activity.

Despite the apparent similarity in the responses to the oxidative stimuli from either side of the membrane, the result that FK-induced anion secretion is inhibited by the apical presence of H2O2 but potentiated by its basolateral presence led us to hypothesize that H2O2 induces different signal transductions in each membrane. The effect of apical H2O2 is reasonable because the ISC changes correlated with FK-induced ICl and [cAMP] elevation. In the hindrance of cAMP production, oxidative stress may operate primarily at the level of the plasma membrane on the functioning of adenylate cyclase by altering the state of phosphorylation (See et al., 2001). However, the effect of H2O2 is also observed in the apical ICl in response to 8-Br-cAMP, a cell-permeable cAMP analog, suggesting that oxidative stress to either aspect hinders activation of CFTR by hindrance of the channel gating and cAMP synthesis. Regarding the mechanisms underlying dysfunction of CFTR under oxidative insults, previous studies have shown that redox reagents alter the kinetics of CFTR gating such that reducing conditions speed up gating and increase the open probability, whereas oxidizing conditions slow down CFTR gating, probably through cysteine residues located on the nucleotide-binding domains of CFTR (Harrington et al., 1999; Harrington and Kopito, 2002). Alternatively, AMP-activated protein kinase, which is activated by oxidative stress and consequently phosphorylates CFTR to inhibit its conductance, may also be involved in the mechanisms (Walker et al., 2003). Naturally, because FK-induced and 8-Br-cAMP-induced ISC were potentiated by the presence of basolateral H2O2, we first assumed that these ISC-related parameters, such as cAMP production and cAMP-elicited ICl, were correlated with the up-regulated ISC changes. Unexpectedly, however, we found that these parameters were inhibited by basolateral H2O2, inconsistent with the behavior of ISC. These observations led us to conclude that H2O2 stimulation from either side hindered the cAMP/PKA signal transduction (cAMP synthesis process and CFTR activation) from the cytosolic side and simultaneously allowed us to deduce the presence of a specific pathway, which is localized around the basolateral membrane.

**Discussion**

The cells responded to H2O2 from either side of the membrane, generating brief ISC responses that reflect anion secretion. This response seems to involve activation of apically located CFTR Cl⁻ channels because of concomitant increases in ICl that almost reflect augmentation of CFTR-mediated Cl⁻ conductance. Many lines of evidence have shown that the effects of H2O2 on airway muscle contractility are modulated by COX inhibitors or epithelial removal, suggesting the presence of mechanisms to release prostaglandins from airway epithelia in response to ROS (Matyas et al., 2002). As shown in the present study, the marked inhibition of apical or basolateral H2O2-induced ICl by indomethacin suggests that H2O2-induced CFTR-mediated anion secretion seems likely to be, at least in part, mediated by intrinsic prostaglandins depending on COX activity. Furthermore, in the responses, both subtypes of COX seem likely to be involved because ISC responses to H2O2 application were equally suppressed by a COX-2 inhibitor (NS-398, Kᵢ of COX-I/COX-2 = 75/1.77 μM) (Barnett et al., 1994) and a COX-1 inhibitor (SC-560, Kᵢ of COX-I/COX-2 = 9 nM/6.5 μM) (Smith et al., 1998). Although the responses to apical and basolateral H2O2 seem likely to be commonly relevant to COX signals, we found that the ISC responses to apical H2O2 were larger than those to basolateral H2O2. These observations allow us to speculate that polarization of the cells produces laterality between the apical and basolateral membrane in the membrane-located COX activity.

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Anion secretion is the end result of coordinated activities of several different anion transporters. This event requires not only the activity of the apical anion channel but also basolateral transporters (Devor et al., 1999; Ito et al., 2004b). The apical CFTR, which is well accepted as a common pathway for HCO₃⁻ and Cl⁻ export (Devor et al., 1999), displays no less than ~60% of maximum conductance at rest, so that the Calu-3 cell is fully capable of anion secretion even under the cAMP-UNstimulated state (Moon et al., 1997). Thus, rather than CFTR as an anion exit pathway, anion uptake across the basolateral membrane is thought to be the rate limiter that largely determines the overall secretion capacity, as is the case of other polarized epithelia (Matthews, 2002). In epithelial cells, anion entry across the basolateral membrane
the oxidant-induced up-regulation of the NKCC1-mediated transport (Liedtke et al., 2002). Thus, a possible explanation for the seemingly paradoxical results is that \( \text{H}_2\text{O}_2 \)-induced up-regulation of anion uptake across the basolateral membrane would compensate for the down-regulation of CFTR activation of anion exits across the apical membrane. Estimation of the anionic composition of the FK-elicited \( I_{\text{SC}} \) revealed that both bumetanide (an NKCC1 inhibitor) and DNDS (an NBC1/AE2 inhibitor)-sensitive components are similarly increased in the control, whereas the increase evidently favored the bumetanide-sensitive \( I_{\text{SC}} \) in the presence of basolateral \( \text{H}_2\text{O}_2 \) (see Fig. 5, A and B); this suggests that cAMP-activated \( I_{\text{SC}} \) potentiated by the presence of basolateral \( \text{H}_2\text{O}_2 \) mirrored the augmentation of \( \text{Cl}^- \) current mediated through NKCC1, whose sensitivity to PKA may be up-regulated by the basolaterally localized effect of the oxidative stress.

Previous studies (Devor et al., 1999; Ito et al., 2004b) reported that the switch between \( \text{HCO}_3^- \) secretion and \( \text{Cl}^- \) secretion is determined by the basolateral membrane potential regulated by a ChTx-sensitive \( \text{Ca}^{2+} \)-activated \( K^- \) channel, KCNN4. Namely, when the basolateral membrane is hyperpolarized by KCNN4 activation, the driving force for \( \text{HCO}_3^- \) entry via NBC1 (the \( \text{Na}^+/\text{H}^+ \) exchanger), which carries electrogically negative charges into the cell, is reduced, whereas the driving force for \( \text{Cl}^- \) entry across the electroneutral NKCC1 (the \( \text{Na}^+/\text{K}^-/\text{Cl}^- \) cotransporter) is up-regulated. Because the hyperpolarization simultaneously provides a driving force for anion export across the apical CFTR, the activation of the KCNN4 would cause a large \( \text{Cl}^- \) secretion (Moon et al., 1997; Devor et al., 1999). To exclude the possibility of NKCC1 up-regulation by way of KCNN4 activation, we observed the effect of basolateral oxidative stress on the FK-induced responses in the presence of ChTx, but it made no significant difference in the oxidant-induced modulation.

It is now been established that a complex cortical meshwork of cytoskeleton proteins localizes adjacent to the cytosolic faces of the plasma membrane, where it is uniquely placed to interact with a variety of transmembrane proteins such as NKCC1 (Matthews, 2002). For NKCC1 regulation, cAMP may induce the surface recruitment of membrane proteins to form a regulatory complex with NKCC1 (D’Andrea et al., 1996). Furthermore, several lines of evidence have shown that cAMP-dependent signals themselves are transduced to NKCC1, at least in part, by dynamic remodeling of F-actin microfilaments within the cortical submembranous cytoskeleton (Shapiro et al., 1991; Matthews, 2002). It has been shown that exposure to \( \text{H}_2\text{O}_2 \) remodels actin structures that take the form of microfilaments associated with cortical F-actin in Calu-3 cells (Boardman et al., 2004). Thus, it is most conceivable that the oxidant-induced remodeling of the cytoskeleton would help the PKA-induced reorganization of the submembranous cytoskeleton linked to NKCC1, resulting in selective augmentation of bumetanide-sensitive \( I_{\text{SC}} \). Indeed, the oxidant-induced up-regulation of the NKCC1-mediated \( I_{\text{SC}} \) in response to FK was markedly suppressed by Cyto-D. Considering the results obtained from the present study, we propose the hypothetical scheme of \( \text{H}_2\text{O}_2 \)-elicited effects on cAMP-dependent anion secretion shown in Fig. 8.

Collectively, we found that basolaterally but not apically applied \( \text{H}_2\text{O}_2 \) potentiates the subsequent cAMP-mediated \( \text{Cl}^- \) secretion via basolateral NKCC1 whose sensitivities to cAMP/PKA are up-regulated, overcoming the negative effects of \( \text{H}_2\text{O}_2 \) on the apical anion conductance via CFTR. The effects of \( \text{H}_2\text{O}_2 \) localized on the basolateral membrane may be relevant not to basolateral \( \text{Ca}^{2+} \)-activated \( K^- \) channels (KCNN4) but to the basolaterally localized cytoskeleton, which is believed to interact with NKCC1.

Airway epithelial cells are exposed to oxidative stress not only through inhalation of ozone and other environmental oxidants from the apical side but also through intrinsic ROS from the basolateral side because formation of ROS takes places constantly in every cell during the metabolic process (Ricciardolo et al., 2006). Especially in acute and chronic airway inflammations, activated phagocytic cells, such as
neutrophils, eosinophils, monocytes, and macrophages, are recruited in the subepithelial sites of the respiratory tract, and they generate and release large amounts of ROS (Ricciardolo et al., 2006). In contrast to the basolateral membrane, the apical membrane is fully protected by antioxidant substances such as vitamin C and glutathione at very high concentrations in the human airway (Kelly et al., 1999; Dauletbaev et al., 2001). Therefore, the responses shown in our study may serve to compensate for basolateral ROS-induced disturbance of mucociliary clearance to help clear infections before consequent tissue damage can be initiated. The site-dependent effects of H$_2$O$_2$ that we have shown here should provide new insight into a variety of epithelial biology and toxicology in which oxidant stress is implicated.

References


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