Oxidant stress stimulates Ca\textsuperscript{2+}-activated chloride channels in the apical activated membrane of cultured nonciliated human nasal epithelial cells

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Oxidant stress stimulates Ca\textsuperscript{2+}-activated chloride channels in the apical activated membrane of cultured nonciliated human nasal epithelial cells. We show that intracellular hydroxyl radicals directly increase CACC activity. This could be one of the responses of airway epithelial cells to oxidant stress.

METHODS

Culture of human nasal epithelial cells. Profs. A. Coste, P. Herman, and J. Soudant generously provided human nasal mucosa from tubinectomies (20 patients) with informed consent and institutional approval (ethical permission was obtained from the Assistance Publique-Hôpitaux de Paris). Most of the patients were on medication (corticotherapy and/or antibiotic treatments). There were no ciliated cells in 60% of the human turbinate samples. Nasal epithelial cells were cultured by cell outgrowth with a modified explant cell culture technique (8, 31). Nasal mucosa from human turbinate samples was dissected into small sections (1–2 mm\textsuperscript{2}). Three pieces of mucosa were transferred to each 35-mm-diameter Falcon Primaria culture dish, which had been coated with 200 \mu l of 1/10 diluted extracellular matrix product (BTI, Biomatrix I; Clinisciences, Montrouge, France). The explants were cultured in DMEM/Ham’s F-12 without HEPES (1:1; GIBCO, Cergy-Pontoise, France) with 2% Ultroser G (GIBCO), 50 U/ml penicillin, 50 \mu g/ml streptomycin, and 50 \mu g/ml gentamicin (only added to the medium at the beginning of the culture for 2 days) and incubated at 37°C in a humidified atmosphere of 5% CO\textsubscript{2} in air. After 5–15 days in culture, explants were surrounded by a coherent outgrowth of epithelial cells with tight junctions resulting from both cell migration and cell proliferation. The periphery of the outgrowth and the proximal part of the explant contained ciliated and nonciliated cells. Ciliated cells seen at the beginning of the culture migrated with the proliferating nonciliated cells. Ciliary beating and presumptive mucus granules could be seen. The number of ciliated cells decreased with the time of culture. This method of cell culture avoided cell passage and enzyme alteration of ion channels and allowed cell-attached or excised inside-out patch-clamp configurations on the apical cell membrane.

Experimental solutions. To enhance the recording of Cl\textsuperscript{−} channel currents, bath and pipette solutions were nominally free of monova-
lent cations. The bath solution contained (in mM) 145 N-methyl-D-glucamine (NMDG)-Cl\(^-\), 1.4 MgCl\(_2\), 1 CaCl\(_2\), 10 N-tris(hydroxy-methyl)methyl-2-aminoethanesulfonic acid (TES), and 300 mosmol/kgH\(_2\)O. The patch pipettes were filled with solution containing (in mM) 145 NMDG-Cl\(^-\), 2 MgCl\(_2\), 2 CaCl\(_2\), 10 TES, and 300 mosmol/kgH\(_2\)O. The low NMDG-Cl\(^-\) bath solution consisted of (in mM) 15 or 5 NMDG-Cl\(^-\), 1.4 MgCl\(_2\), 1 CaCl\(_2\), 10 TES, and osmolality 300 mosmol/kgH\(_2\)O adjusted with sucrose. The pH of all solutions was 7.4. Solutions were prepared with UltraPure Water (Milli-Q System; Millipore, St-Quentin-en-Yvelines, France). The free Ca\(^{2+}\) concentration in the bath solution was adjusted with Ca\(^{2+}\) and EGTA (Sigma) (19). In recording currents in the cell-attached configuration, the bath solution also sometimes contained 10 \(\mu\)M forskolin (Sigma). Forskolin was dissolved in DMSO, and the final bath concentration of solvent was \(\leq 0.1\%\). For excised, inside-out patch-clamp recording, bath solutions also contained either 150 U/ml catalytic subunit of cAMP-dependent protein kinase (PKA; Promega) and 200 \(\mu\)M MgATP (Sigma) or no additives. Chemicals employed were of the highest grade of purity available (Merck, Darmstadt, Germany; Pro-labo, Fontenay-sous-Bois, France; Sigma Aldrich). CaM human brain (CaM), Ca\(^{2+}/CaM\) kinase II, rat brain (CaMK), and Ca\(^{2+}/CaM\) kinase II inhibitor peptide 281-309 (CaMKI) were obtained from Calbiochem (Merck Biosciences, Nottingham, UK). The reduced form of glutathione was obtained from Sigma.

**Electrophysiology.** Ion channel currents were recorded with the patch-clamp technique in cell-attached and inside-out membrane patch configurations performed on the apical membrane of human nasal nonciliated cells from the periphery of the cell outgrowth layer. Strong outward rectification and a high rate of channel activation were observed in the pipette solution. This method avoided enzymatic treatment of the cells. The bath solution surrounding cells or excised membrane patches could be changed with a gravity-driven, multibarrel perfusion system (7 reservoirs) placed within 100 \(\mu\)m of the pipette patch and delivering 34 \(\mu\)l/min. Solution changes were achieved within 10 s by manual switching between reservoirs. Recording pipettes were pulled in two stages from borosilicate glass capillary tubes (GC 150-7.5; Clark Electromedical Instruments, Reading, UK). Pipettes were coated with two layers of Sylgard 184 (Dow Corning Europe, Brussels, Belgium) and fire-polished and had a resistance of 15–20 M\(\Omega\) when filled with the pipette solution. The reference Ag-AgCl electrode was connected to the bath via an NMDG-Cl\(^-\) agar bridge. Single-channel currents were recorded with an Axopatch 200B amplifier (Axon Instruments, Dipsi Industrie, Chatillon-sous-Bagneux, France), filtered using a four-pole Bessel filter at 1 kHz and recorded on digital audiotape (DAT DTR, 1205; Biologic, Claix, France). Current recordings were converted using an analog-to-digital interface (DIMA 100 OEM, Card Lab Master, Biologic) to a computer running appropriate software (pCLAMP, v.6; Axon Instruments, Foster City, CA). Currents were digitized at 20 kHz. Recording sequences (30 or 60 s or several minutes) were chosen upon replay of DAT cassettes then transferred to storage media (ZIP disks; Iomega, Ropy, UT) or to a printer (Dash IV model XL; Astro-Med, Trappes, France) for long sequences. Experiments were performed at room temperature (21–23°C).

**Data analysis.** Single-channel data were analyzed using pCLAMP software (v.6). Channels were identified and characterized according to their ionic selectivity with respect to a NMDG-Cl\(^-\) concentration gradient (15 mM in the bath vs. 145 mM NMDG-Cl\(^-\) in the pipette) and their single-channel conductance. Unitary current reversal potential and conductance values were estimated from the linear portion of current-voltage (I-V) relationships. Channel amplitude was calculated from Gaussian fits to amplitude/distribution histograms constructed from single-channel recordings. The probability of a channel being open (\(P_o\)) was measured from 30- to 60-s stable and representative recordings. To calculate \(P_o\), digitized single-channel data were subjected to event detection (pCLAMP). \(P_o\) was calculated as the fraction of the specified recording time spent by the channel in the open state.

**RESULTS**

**Ca\(^{2+}/CaM\)-dependent, protein kinase II-dependent CACC in apical membranes of nonciliated human nasal epithelial cells.** In 98 cell-attached patches (Fig. 1) and 74 inside-out patches (Fig. 2), we recorded channel currents characterized by a strong outward rectification and a high rate of channel activation with depolarization. The unitary I-V relationship obtained from these channels in cell-attached patches in symmetrical NMDG/Cl\(^-\) solutions was rectified over the range -140 to +140 mV (see Fig. 4, \(n = 23\)). The mean conductance of the channels was 5.2 ± 0.5 pS at +120 mV and 2.8 ± 0.3 pS at -100 mV. The reversal potential was at or close to 0 mV in symmetrical 145 mM NMDG/Cl\(^-\) solutions in 21 out of 23 cell-attached patches. The I-V relationships from cell-attached and inside-out patches in symmetrical 145 mM were similar (see Fig. 4, \(n = 23\) and \(n = 15\), respectively) with the same rectification and the same conductance (5.3 pS at +120 mV and 1.9 pS at -140 mV in inside-out patches). Under asymmetrical conditions, when intracellular [NMDG/Cl\(^-\)] was reduced to 15 mM (Figs. 3A and 4), a negative reversal potential was measured (−52 ± 5 mV). This value is close to the −56 mV predicted by the Goldman-Hodgkin-Katz voltage equation for a perfectly anion-selective channel. In asymmetric Cl\(^-\) solutions, the single-channel conductance was significantly increased (11 pS at +100 mV, \(P < 0.05\)), and the recordings and the magnitude of the channel currents between ±100 mV were improved (Fig. 3A) compared with 145 mM NMDG/Cl\(^-\) symmetrical conditions. When intracellular [NMDG/Cl\(^-\)] was
reduced to 5 mM, only a negative current was observed between $-140$ and $-60$ mV. These experimental conditions favor the recording of anionic channel currents, and separate experiments showed that these channels were more permeant to I$^-$ and Br$^-$ than Cl$^-$ (data not shown) and thus represent an anion-selective channel.

In cell-attached membrane patches, chloride channel activity was not sustained but declined and disappeared within several
minutes of the onset of recording. This rundown of channel activity was independent of the type of bathing solution whether this contained forskolin and ATP \((n = 57)\), ATP alone \((n = 4)\), or no additives to the NMDG/Cl\(^-\) bath solution \((n = 41)\). Activity could be regained with a second application of forskolin following a period of washing \((n = 21)\).

In cell-attached or inside-out patches in symmetrical or asymmetric NMDG/Cl\(^-\) bath solution, the chloride channel activity was voltage dependent, increasing with depolarization of the membrane (Figs. 1, 2, and 3A). In inside-out patches containing only one channel at hyperpolarized membrane potential under asymmetric Cl\(^-\) conditions \((15 \text{ mM NMDG/Cl}^-\)
Fig. 3. In inside-out patches, although these low-conductance Cl⁻ channels could be activated by membrane depolarization, this effect gradually declined, with rundown being complete 3–5 min after excision.

Figure 5A shows single-channel current recording from an excised inside-out membrane patch containing two channels. At +100 mV, the open channel currents were outwardly directed. Reducing [Ca²⁺] in the bath solution from 10⁻³ to 10⁻⁷ M decreased Pₒ from 0.56 ± 0.06 to 0.05 ± 0.01 (n = 3, P < 0.05), and channel activity ceased completely at 10⁻⁸ M [Ca²⁺] (Fig. 5B). This effect was rapidly reversible, and recovery of channel activity was obtained by increasing [Ca²⁺] in the bath from 10⁻⁸ to 10⁻³ M (n = 5). These results suggest that these channels belong to the CACC family.

In some experiments, single-channel current recordings from inside-out patches were held at +140 mV in solutions containing 10⁻³ M Ca²⁺ until channel opening disappeared 2–5 min after excision. Then, the addition of CaM alone (5 or 20 µg/ml), ATP alone (500 µM), or CaM and ATP had no effect. But the addition of CaM kinase II (0.08 µg/ml) in the presence of Ca²⁺, CaM, and ATP led to channel activation (Fig. 6A). These Ca²⁺/CaM kinase II-activated channels showed outward rectification, and their conductance was similar to that shown in Fig. 2. This effect of CaM kinase II was blocked by the application of 3 mM CaM kinase II inhibitory peptide (281-309) (Fig. 6B). These observations were reproduced in three patches.

In summary, these experiments show that voltage- and Ca²⁺-activated chloride channels (CACC) with outwardly rectified low conductance could be activated by Ca²⁺/CaM kinase II in the apical membrane of nonciliated human nasal epithelial cells.

Effects of exposure of the cytoplasmic face of the CACC to -OH. H₂O₂ is converted to short-lived -OH when mixed with Fe²⁺ (17). The simultaneous application of H₂O₂ (10⁻² M) and Fe²⁺ (10⁻⁵ M) produced -OH continuously in front of the patch pipette (for several minutes), and this increased Pₒ to 0.26 ± 0.05 and 0.67 ± 0.06 at +140 mV (Fig. 7, B and C, n = 17). The effect of -OH was sustained following return to control bath solution that contained either 10⁻³ M or 10⁻⁸ M [Ca²⁺] (Fig. 7C). The magnitude of the effect of -OH was similar to that which had been evoked by Ca²⁺/CaM kinase/ATP. The effects of -OH on CACC were also tested in solutions containing 10⁻⁸ M [Ca²⁺]. Pₒ increased from 0 to 0.50 after a 1-min exposure to -OH at a membrane potential of +140 mV (n = 3). The I-V relationship of the -OH-activated channel was the same as that of the Ca²⁺-activated channel with equivalent rectification and conductance. Channel activity evoked after brief exposure to -OH (1 min, 10⁻⁵ M Fe²⁺ and 10⁻² M H₂O₂) was reversibly inhibited by the simultaneous application of the antioxidant DMTU (Fig. 8C). The simultaneous application of Fe²⁺ (10⁻⁵ M) and variable H₂O₂ concentrations (0.02, 0.04, 0.1, 0.2, 0.4, 0.5, 1, 2, 5, 8, and 10 mM in the superfusion system) produced increasing quantities of hydroxyl radicals and a dose-dependent effect of -OH on channel activity (Table 1 and Fig. 9). The increase in Pₒ was reversible spontaneously without wash out when we applied from 0.02 to 1 mM H₂O₂ and 10 µM Fe²⁺. The dose-response effect of -OH is shown in Table 1. The effect of -OH on the CACC activity was high and sustained when we applied 2–8 mM H₂O₂ (Table 1 and Fig. 9) and decreased upon either the wash out of H₂O₂ (Fig. 9D) or the addition of 3 mM CaMKI (Fig. 9E). It was inhibited by 1.5 mM GSH (Fig. 9F). Variability in the duration of exposure to -OH before channel activation, magnitude of Pₒ, and duration of channel activity, probably reflected the different redox status of different cells (Table 1). The effect of the highest concentration of -OH (10 mM H₂O₂) on the channel activity was sustained for several minutes and irreversible following return to control bath solution with
10^{-3} \text{ M} \text{ or } 10^{-8} \text{ M} [\text{Ca}^{2+}] \) (see Fig. 7C). In this case, reversibility could only be evoked with 1.5 mM GSH (3 patches).

**Effects of extracellular forskolin and \cdot\text{OH on CACC activity in cell-attached patches.** Exposure of intact cells to forskolin (10 \mu M) elicited, after a short delay (1 min), a marked and reversible increase in channel \( P_\alpha \) in 21 cell-attached patches tested. The application of \cdot\text{OH} to intact cells also elicited, after a short delay (1–2 min), a reversible increase in channel \( P_\alpha \) in cell-attached patches (\( P_\alpha = 0.57 \pm 0.05, n = 7 \)).

**DISCUSSION**

This study shows for the first time that a CACC can be both reversibly and irreversibly activated by exposure to \cdot\text{OH}. The
Fig. 6. Effects of Ca\textsuperscript{2+}/calmodulin (CaM) kinase II and Ca\textsuperscript{2+}/CaM kinase II inhibitor peptide (281-309) on Cl\textsuperscript{-} channel activity. A: example of current recording from an inside-out patch with 145 mM NMDG/Cl\textsuperscript{-} and 10^{-3} M Ca\textsuperscript{2+} in the bath and pipette solutions, 5 min after excision after loss/rundown of Cl\textsuperscript{-} channel activity. When the bath solution was changed for one that contained 0.08 \mu g/ml CaM kinase II, 5 \mu g/ml CaM, 500 \mu M ATP, and 10^{-3} M Ca\textsuperscript{2+}, 2 channels were activated 3 min after addition of the 3 compounds. Data were recorded at a Vm of +140 mV. B: a different recording from an inside-out patch, 2 min after excision in symmetrical 145 mM NMDG/Cl\textsuperscript{-} and 10^{-3} M Ca\textsuperscript{2+} with no additives in the bath. In this example, the channels, which had been stably activated by membrane depolarization to +120 mV, closed within 30 s of exposure to 3 nM Ca\textsuperscript{2+}/CaM kinase II inhibitor peptide (281-309). These observations were reproduced in 3 patches.

Fig. 7. Effects on channel $P_o$ of exposure of the cytoplasmic face to hydroxyl radicals. A: current recording from an inside-out patch in symmetrical 145 mM NMDG/Cl\textsuperscript{-} bath solution containing 10^{-3} M Ca\textsuperscript{2+} that showed the loss/rundown of Cl\textsuperscript{-} channel activity 5 min after excision. Data were recorded at a Vm of +140 mV. B: Cl\textsuperscript{-} channels were activated 20 s after exposure to -OH (10^{-6} M Fe\textsuperscript{3+} and 10^{-2} M H\textsubscript{2}O\textsubscript{2}). These observations were reproduced in 17 patches. C: irreversible activation of Cl\textsuperscript{-} channel activity 2 min after exposure to -OH and 1 min wash out with 145 mM NMDG/Cl\textsuperscript{-} bath solution containing 10^{-8} M Ca\textsuperscript{2+}. These observations were reproduced in 7 patches.
same channel was activated upon exposure of intact cells to 
-HOH. These observations have pathophysiological implications
for human nasal epithelial cells. The reversible and irreversible
activation of CACC in excised patches by oxidative stress
without change in intracellular [Ca2+] is a new observation.
The reversibility depended on the quantity of hydroxyl radicals
produced according to the H2O2 concentrations superfused.
Because of its low oxidizing potential, H2O2 is not by itself
usually reactive enough with organic molecules. Nevertheless,
H2O2 has the ability to generate highly reactive
-HOH through its
interaction with redox-active transitional metals. PM2.5, which
is incriminated in respiratory disorders, is characterized by
high Fe and Cu contents. The suspension of PM in water
(100–1,000 μg/ml) released hydroxyl radicals in the absence
or in the presence of H2O2 (3). Therefore, metals adsorbed on
PM become bioavailable and generate -OH with or without
H2O2 in ranges comparable with those of this study (3).
Here, we show that applying H2O2 simultaneously with
Fe2+ to the cytoplasmic face of excised inside-out mem-
brane patches of human nasal cells produced a dose-depen-
dent increase in Po of CACC. This suggests that
-HOH could
be targeting exposed cysteine SH residues. The redox con-
dition of these SH groups changes with the addition of GSH.
These SH residues could be located near the Ca2+/CaM
kinase site on the channel because CaMKI also decreased
channel activity.

Table 1. Dose-response relationship between 10 μM Fe2+ with different [H2O2] and the effect of -OH on CACC activity

<table>
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<tr>
<th>[H2O2], mM</th>
<th>n</th>
<th>Duration (s) of exposure to -OH before channel activation (range)</th>
<th>Po (1 channel or level 1) means ± SE</th>
<th>Duration (s) of channel activity (range)</th>
<th>Number of channels in patch (range)</th>
<th>Reversibility</th>
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<tr>
<td>0</td>
<td>10</td>
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<td>0.02±0.01</td>
<td>0.5-2</td>
<td>1</td>
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<tr>
<td>0.02</td>
<td>3</td>
<td>5-40</td>
<td>0.07±0.09</td>
<td>8-90</td>
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<td></td>
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<tr>
<td>0.04</td>
<td>4</td>
<td>25-120</td>
<td>0.23±0.03</td>
<td>25-90</td>
<td>3</td>
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<tr>
<td>0.10</td>
<td>5</td>
<td>15-120</td>
<td>0.33±0.15</td>
<td>8-120</td>
<td>2</td>
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<tr>
<td>0.20</td>
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<td>50-180</td>
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<td>0.40</td>
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<tr>
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<tr>
<td>1</td>
<td>4</td>
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<td>0.28±0.07</td>
<td>70-120</td>
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<td>6</td>
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<td>0.36±0.15</td>
<td>10-120</td>
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<tr>
<td>5</td>
<td>4</td>
<td>70-120</td>
<td>0.56±0.08</td>
<td>&gt;120</td>
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</tr>
<tr>
<td>8</td>
<td>2</td>
<td>5-40</td>
<td>0.50±0.10</td>
<td>&gt;120</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>8</td>
<td>5-300</td>
<td>0.70±0.02</td>
<td>&gt;120</td>
<td>4</td>
<td>Only with GSH</td>
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Effects of extracellular forskolin and -OH on CACC activity in cell-attached patches. Po, open probability; CACC, Ca2+-activated Cl- channel. Membrane potential = +120 mV; n = number of experiments.
An effect of ROS on a Cl\(^{-}\) current has also been described for transepithelial anion secretion across monolayers of human submucosal gland serous cells (7). They suggest that CFTR is the major anion conduction pathway mediating this response and that this plays an important role in keeping the airways clear from damaging radicals that could potentially initiate tissue destruction. H\(_2\)O\(_2\) has also been described as an essential second messenger mediating the activation of volume-sensitive ORCC in HTC and HeLa cell lines (29). ROS also increase mucus secretion from rodent respiratory epithelial cells through a mechanism involving cyclooxygenase metabolism of arachidonic acid with production of PGF\(_2\)\(\alpha\) (1). Our results suggest that when the intracellular protective mechanisms against oxidants are overloaded in human nonciliated nasal...
epithelial cells, CACC located on the apical membrane are activated by ROS.

This is the first description of a CACC in nonciliated human nasal epithelial cells. CACC have also been reported in rat lacrimal gland secretory cells (23), in normal and cystic fibrosis human nasal epithelia mounted in Ussing chambers (5), in a distal nephron A6 cell line (24), and in guinea pig hepatocytes (18). In all cases, the low conductance of the channels or current showed strong outward rectification, and channel P0 was increased by depolarization and [Ca2+]i. We show that this channel was also regulated by Ca2+ acting via a CaM kinase II-dependent mechanism. Cl− channel activation by Ca2+ mediated by multifunctional Ca2+/CaM-dependent protein kinase has also been found in human airway cell lines (30).

There are 12 genes in the CACC gene family (11). The expression of the human (h) CACC2 and hCACC3, but not hCACC1, was demonstrated in human native nasal tissues by RT-PCR (21). On the other hand, the porcine pCACC1 gene, which has 78% amino acid sequence identity with hCACC1, contains a unique A-kinase consensus site on the cytoplasmic loop between the putative transmembrane TM3 and TM4 domains, and expression in an epithelial cell line revealed a CACC that could be activated by cAMP (20). The CACC described in the present study was also activated by cAMP. That cAMP activates basolateral K+ channels, which could generate the driving force for CACC-mediated Cl− secretion in epithelia (21), cannot account for activation of CACC in excised membrane patches.

Notwithstanding that forskolin, PKA, and ATP were added together or separately in the bath solution applied to intact cells and inside-out membrane patches, we failed to record CFTR. Immunohistochemical studies have localized CFTR to the apical domain of ciliated cells in epithelia from human fetal airway, human nasal polyps, and human adult turbinates mucosa (12, 15, 28). It is therefore perhaps not surprising that we have not found CFTR-like ion channels in the apical membrane of nonciliated human nasal epithelial cells. However, it should be noted that extracellular nucleotides can regulate anion transport in airway epithelia (25). This effect is mediated by plasma membrane P2Y receptors, activation of phospholipase C, the generation of d-myoinositol 1,4,5-trisphosphate, and mobilization of Ca2+ from internal stores. Apical CACC are then stimulated via an increase of intracellular [Ca2+]i (9, 14, 27). This phenomenon may have been observed in cell-attached membrane patches in the present study since ATP was present in the bath solution at the beginning of some experiments.

In conclusion, we have shown that CACC were reversibly or irreversibly activated by intracellular exposure to OH without change in intracellular [Ca2+]i. Reversibility depended on the H2O2 concentration and the duration of exposure to OH. This was blocked by the antioxidant DMTU after a brief exposure to OH generated by the highest H2O2 concentration. These results suggest a pathophysiological role for OH that could lead to sustained inflammation of the airway.

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