Hydroxyl radical activation of a Ca\(^{2+}\)-sensitive nonselective cation channel involved in epithelial cell necrosis

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Hydroxyl radical activation of a Ca\(^{2+}\)-sensitive nonselective cation channel involved in epithelial cell necrosis. Am J Physiol Cell Physiol 287: C963–C970, 2004. First published May 26, 2004; 10.1152/ajpcell.00041.2004.—In a previous work the involvement of a fenamate-sensitive Ca\(^{2+}\)-activated nonselective cation channel (NSCC) in free radical-induced rat liver cell necrosis was demonstrated (5). Therefore, we studied the effect of radical oxygen species and oxidizing agents on the gating behavior of a NSCC in a liver-derivated epithelial cell line (HTC). Single-channel currents were recorded in HTC cells by the excised inside-out configuration of the patch-clamp technique. In this cell line, we characterize a 19-pS Ca\(^{2+}\)-activated, ATP- and fenamate-sensitive NSCC nearly equally permeable to monovalent cations. In the presence of Fe\(^{2+}\), exposure of the intracellular side of NSCC to H\(_2\)O\(_2\) increased their open probability (\(P_o\)) by \(\sim 40\%\) without affecting the unitary conductance. Desferrioxamine as well as the hydroxyl radical (OH) scavenger MCI-186 inhibited the effect of H\(_2\)O\(_2\), indicating that the increase in \(P_o\) was mediated by OH. Exposure of the patch membrane to the oxidizing agent 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) had a similar effect to -OH. The increase in \(P_o\) induced by -OH or DTNB was not reverted by preventing formation or by DTNB washout, respectively. However, the reducing agent dithiothreitol completely reversed the effects on \(P_o\) of both -OH and DTNB. A similar increase in \(P_o\) was observed by applying the physiological oxidizing molecule GSSG. Moreover, GSSG-oxidized channels showed enhanced sensitivity to Ca\(^{2+}\). The effect of GSSG was fully reversed by GSH. These results suggest an intracellular site(s) of action of oxidizing agents on cysteine targets on the fenamate-sensitive NSCC protein implicated in epithelial cell necrosis.

Ca\(^{2+}\)-activated channels; radical oxygen species; oxidative stress

RADICAL oxygen species (ROS) play an essential role in many physiological and pathological processes (43). In physiological conditions, it is now well documented that addition of exogenous H\(_2\)O\(_2\) or increased intracellular generation of H\(_2\)O\(_2\) influences the function of various proteins, including protein tyrosine phosphatases, protein kinases, and transcription factors, suggesting that H\(_2\)O\(_2\) and possibly other ROS act as intracellular messengers (18, 43, 48). Intracellular accumulation of ROS, such as H\(_2\)O\(_2\), superoxide anion, and hydroxyl radical (OH), results from normal metabolic processes or toxic insults. The degree of oxidative stress, which may potentially lead to cell damage, is determined by the balance between free radical synthesis and degradation (19). Alterations of this balance have been associated with several pathological processes like inflammation, aging, and ischemia-reperfusion. In addition, high levels of ROS can cause necrosis (14, 35), whereas lower levels can trigger apoptosis (11, 14, 23). Direct treatment of cells with oxidants has been demonstrated to induce necrosis, which appears to be the result of acute cellular dysfunction in response to severe stress conditions or after exposure to toxic agents. In opposition to apoptosis, necrosis is characterized by rapid cellular ATP depletion and a significant increase in cell volume, termed necrotic volume increase (38). Although necrotic cell swelling has been associated with defective outward Na\(^{+}\) pumping in low-ATP conditions, several studies now indicate that the observed Na\(^{+}\) overload is due to an increase in cell membrane Na\(^{+}\) permeability attributable to the activation of nonselective cation channels (NSCC) (5, 12, 44).

Furthermore, in energized cells, ouabain-mediated inhibition of the Na\(^{+}\)-K\(^{+}\)-ATPase pump does not produce cell death (7, 29), implicating mechanisms other than pump failure as critical to swelling of necrotic cells.

Fenamate-sensitive NSCCs that are activated by intracellular Ca\(^{2+}\) and inhibited by intracellular ATP have been identified in several cell types, both native and cultured (5, 9, 12, 15, 21, 22, 33, 42, 45). These channels exhibit single-channel conductances in the range of 15–35 pS, discriminate poorly between Na\(^{+}\) and K\(^{+}\), and are impermeable to anions and, for the most part, to divalent cations. In addition, NSCC are blocked by the adenosine nucleotides ATP, ADP, and AMP on the cytoplasmic side. The physiological role of these channels remains unclear, in part because of the high concentrations of intracellular Ca\(^{2+}\) that are generally required for channel activation.

Redox modulation of ion channels is not without precedent. The large-conductance Ca\(^{2+}\)-sensitive voltage-dependent K\(^{+}\) (K\(_{V,\text{Ca}}\)) channel from tracheal myocytes is inhibited by oxidizing agents (49). A similar result was observed in reconstituted skeletal muscle K\(_{V,\text{Ca}}\) channels incorporated into bilayers (46). In contrast, ryanodine receptor/Ca\(^{2+}\) release channel activity is enhanced by endogenous oxidizing molecules (2, 16). Anion channels are also sensitive to redox modulation. The outwardly rectifying chloride channel in bronchial epithelial cells was shown to be irreversibly inhibited by long exposure to -OH on the cytoplasmic side of the channel (28). Activation of NSCCs by ROS, including superoxide anion, -OH, and H\(_2\)O\(_2\), has been reported in different cell types (5, 24, 27, 31, 33, 36). Furthermore, cells exposed to severe stress conditions exhibit significant ATP depletion and intracellular Ca\(^{2+}\) increase, which is paralleled by an increase in ROS. Under these conditions, NSCC would activate and thus participate in the cation flux involved in necrotic cell swelling (5).
In the present study, we have characterized a NSCC of liver-derived epithelial (HTC) cells and examined more closely the mode of action of H₂O₂ on this channel. We found that the H₂O₂ effect on the NSCC channel activity in HTC cells is mediated by the formation of -OH, which most probably targets residues located at the intracellular aspect of the NSCC channel. We also found that the effects of H₂O₂ are mimicked by GSSG, a physiological molecule that specifically reacts with sulfhydryl (SH) groups, leading to the conclusion that redox modulation most probably involves a disulfide/thiol exchange of thiol groups of cysteines that may be present in the NSCC protein. In addition, oxidation by GSSG shifts the Ca²⁺ vs. open probability (Pₒ) curve to the left, indicating that redox modulation of NSCC may play a significant role in signaling mechanisms leading to necrotic cell volume increase and cell demise.

MATERIALS AND METHODS

Cell culture and electrophysiological measurements. HTC cells were grown at 37°C in a 5% CO₂-95% air atmosphere in DMEM (GIBCO, Grand Island, NY) supplemented with 5% fetal calf serum (GIBCO), 2 mM l-glutamine, 50 U/ml penicillin-streptomycin, and 2.5 µg/ml amphotericin B (Sigma, St. Louis, MO). For electrophysiological experiments, cells were grown on 22-mm coverslips and directly mounted on the experimental chamber (RC-25, Warner Instruments) installed on the stage of an inverted microscope (IX-70, Olympus America or Nikon Diaphot, Nikon America). Solution changes were done by a gravity-fed perfusion system, and the solution level in the chamber was kept constant by a peristaltic pump. To avoid contamination from anion currents, all experiments were carried out in low-chloride solutions, unless otherwise indicated. The bath (intracellular) high-Ca²⁺ solution contained (in mM) 145 sodium glutamate, 2.6 CaCl₂, 1.3 MgCl₂, 5.6 glucose, and 10 HEPES, pH 7.4, adjusted with Tris. The Ca²⁺-free solution contained (in mM) 145 sodium glutamate, 1.3 MgCl₂, 2 EGTA, 5.6 glucose, and 10 HEPES, pH 7.4, adjusted with Tris. Experiments with oxidizing agents were carried out in a 300 µM free Ca²⁺ solution containing (in mM) 145 sodium glutamate, 1 nitrolitriacetic acid trisodium salt, 0.863 CaCl₂, 1.56 MgCl₂, 5.6 glucose and 10 HEPES, pH 7.4, adjusted with Tris. At this intracellular Ca²⁺ concentration ([Ca²⁺]; [Ca²⁺]), small changes in Pₒ were clearly resolved (see Fig. 2B). The free [Ca²⁺] of solutions used was calculated using the program WinMaxv, version 2.05 (www.stanford.edu/~capton/maxv.html), with the appropriate Ca²⁺ buffers. The pipette (extracellular) solution, unless indicated otherwise, contained (in mM) 145 sodium glutamate, 2.6 CaCl₂, 1.3 MgCl₂, 5.6 glucose, and 10 HEPES, pH 7.4, adjusted with Tris. Cation selectivity of the NSCC was demonstrated using asymmetrical Na⁺ solutions. In relative permeability experiments for monovalent cations, internal Na⁺ was substituted by equimolar amounts of K⁺, Rb⁺, and Cs⁺. Relative permeability for divalent cations was studied by replacing sodium glutamate in the pipette with 75 mM CaCl₂ or 75 MgCl₂. Changes in liquid junction potential were calculated (6) and voltages were corrected for, when necessary. Patch-clamp pipettes were made from thin borosilicate (hard) glass capillary tubing with an outer diameter of 1.5 or 1.7 mm (Clark Electromedical, Reading, UK), using a BB-CH puller (Mecanex, Geneva, Switzerland). Single-channel currents were recorded with an EPC-7 (List Medical, Darmstadt, Germany) or an Axopatch B (Axon Instruments) amplifier. An agar bridge connected the reference electrode to the bath. Command voltage protocols and single-channel current acquisition were controlled by pCLAMP 8 (Axon Instruments) via a laboratory interface (Digidata 1200 or Digidata 1322A, Axon Instruments). Unless otherwise indicated, membrane holding potential (Vₘₚ) was −60 mV.

Currents were filtered at 1 kHz with an 8-pole Bessel filter (Frequency Devices) and digitized at 5 kHz. The experiments were performed at room temperature. The number of channels in a given patch, N, was experimentally determined at the beginning of each experiment by exposing the membrane patch to 2.6 mM Ca²⁺ followed by Ca²⁺-free solution (see RESULTS and Fig. 2B). This figure (N) was used to calculate Pₒ. The number of channels was frequently ~6 (range 3–16). When possible, N was also determined at the end of the experiment. In these cases N remained unchanged.

Data analysis. Current analysis was performed off-line using pCLAMP 8 (Axon Instruments) and SigmaPlot 5.0 (Jandel, Erkraft, Germany). Data for analysis (60–120 s) were taken when steady state was achieved (at least 120 s after the bath solution was changed; see stability plots in Figs. 4, 6, and 7). All-points histograms were generated using QuB software (40, 41). Estimation of single-channel current amplitude was obtained by fitting a multiple Gaussian function to the data and averaging the differences between consecutive means of each Gaussian. Single-channel conductance was estimated by fitting a linear regression to the data in symmetrical ionic conditions. In asymmetrical ionic conditions the Goldman-Hodgkin-Katz (GHK) current equation was fitted to the data (25). Permeability ratios (Pₛ/Pₓ) for monovalent cations were estimated from the GHK current equation fitted to the data. Pₒ was calculated by assuming N independent channels by fitting the amplitude histograms to the binomial function (17):

\[
P(N,x,Pₒ) = \binom{N}{x} (Pₒ)^x (1-Pₒ)^{N-x}
\]

where xstands for open channels from x = 0 to N. In addition, the error associated with the estimation of the total number of channels in the patch was calculated using a minimum square error function of the form

\[
\text{error}(P) = \sum_{x=1}^{N} [P(N,x,Pₒ) - A_x]^2
\]

where Aᵢ corresponds to the area of open-state amplitude histograms from x = 1 to N. For all experiments, the minimum error always corresponded to the same value of N determined experimentally.

-ÖH generation. The cytoplasmic side of the patch membrane was continuously superfused with two 300 µM Ca²⁺ intracellular solutions containing 10 mM H₂O₂ and 10 µM FeSO₄, respectively, by using independent, light-protected local perfusion systems, yielding an estimated final H₂O₂ concentration of 5 mM and FeSO₄ concentration of 5 µM. Working solutions were prepared at the moment of the experiment. The mixture produces ÖH according to the Fenton reaction. The generation of ÖH in the bath solution was confirmed by electron spin resonance spectroscopy as described (28). Briefly, the burst of ÖH was detected utilizing 5,5'-dimethyl-1-pyrroline N-oxide (DMPO) as a spin-trapping agent. The electron paramagnetic resonance spectra from the long-lived (DMPO-ÖH) spin adducts produced in the bath solution were measured in a spectrometer (Bruker ECS 106, Ettlingen, Germany).

Reagents. All reagents were of analytical grade and were purchased from Sigma and Merck (Darmstadt, Germany). MCI-186 was purchased from Calbiochem (San Diego, CA).

Statistics. Data are presented as means ± SE. Statistical analysis of the data was performed by paired and unpaired Student’s t-test and was considered significant at P < 0.05. One-way ANOVA test was performed for multiple treated samples and was considered significant at P < 0.05.

RESULTS

Characterization of NSCC in HTC cells. NSCC have been found in different types of liver-derived cell lines and, although they share some functional properties, they differ in their Ca²⁺
Permeation properties of nonselective cation channels (NSCC). Experiments were carried out in the presence of 2.6 mM intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)).

A: unitary outward and inward currents recorded at the indicated membrane holding potential (V\(_m\)) with symmetrical (145 Na\(^+\)/H11001, ○) and asymmetrical (40/145, [Na\(^+\)/H11001]\(_i\)/[Na\(^+\)/H11001]\(_o\) , □) solutions. Arrows point toward channel closures.

B: current-voltage (I-V) relationships of NSCC. Solutions were the same as described above. Slope conductance and reversal potential (V\(_{rev}\)) were 18.9 ± 0.04 pS and −0.5 ± 0.9 mV, respectively, for symmetrical Na\(^+\) (○, n = 25). In asymmetrical Na\(^+\) (□, n = 4), the continuous line represents a Goldman-Hodgkin-Katz (GHK) current equation fitted to the data.

C: unitary outward currents obtained at V\(_m\)/50 mV for the different monovalent cations (Cl\(^–\)/salt) tested. Arrows point toward channel closures.

D: I-V relationships of unitary NSCC currents obtained in 145 mM external sodium glutamate and 145 mM internal XCl, where X stands for the different monovalent cations used: K\(^+\) (○), Rb\(^+\) (□), and Cs\(^+\) (△) (n = 10–15). Solid lines represent GHK current equation fitted to the data; permeability ratios are shown in the text. Data are expressed as means ± SE. Error bars are shown if bigger than symbols.

Fig. 2. Calcium modulates NSCC activity. A: single-channel activity recorded from a representative inside-out patch containing N = 3 channels exposed to different free [Ca\(^{2+}\)]. From top to bottom: 0, 0.1, 0.3, 1, 2.6, and 10 mM free [Ca\(^{2+}\)]. Dashed lines indicate zero current (C); O\(_x\) represents the level of current carried by x channels. B: Ca\(^{2+}\) dependence of open probability (P\(_o\)). Each membrane patch was exposed to all Ca\(^{2+}\) concentrations (n = 4). Continuous line represents Hill equation fitted to the data with n = 1.54 ± 0.15 and EC\(_{50}\) = 455 ± 33 μM. C: effect of [Ca\(^{2+}\)] on the unitary conductance of NSCC. The number of experiments is shown in parentheses above symbols. Continuous line represents Michaelis-Menten equation fitted to the data with maximum single-channel conductance (γ\(_{max}\)) = 23.6 ± 0.6 pS and K\(_d\) = 10.1 ± 0.8 mM. Data are expressed as means ± SE. Error bars are shown if bigger than symbols.
permeability (5, 20, 32). In the cell-attached configuration with a pipette solution containing 145 mM sodium glutamate, very few channel openings were observed. After patch excision (inside-out) into a 145 mM sodium glutamate- and Ca\(^{2+}\)-containing bath solution, channel activity increased significantly, revealing multiple level single-channel currents that were blocked by 100 \(\mu\)M flufenamic acid (not shown; Ref. 45). Experiments showing inward and outward single-channel currents obtained at 2.6 mM [Ca\(^{2+}\)], and symmetrical sodium glutamate (Fig. 1A) were used to construct the current-voltage relationship over the range -80 to 60 mV, depicted in Fig. 1B. The linear fit shows that current reversed close to 0 mV with a slope conductance of 18.9 ± 0.4 pS. Under these experimental conditions, \(P_o\) was close to 1 (Fig. 2B) and was voltage independent (not shown). In low (40 mM) internal Na\(^+\), the current followed the GHK formalism expected for a cation-selective channel (Fig. 1B). To test channel selectivity for different monovalent cations, internal Na\(^+\) was replaced by equimolar concentrations of K\(^+\), Rb\(^+\) and Cs\(^+\) (Fig. 1, C and D). These replacements did not significantly change unitary current amplitude. The relative permeability (\(P_o\),/\(P_x\)) sequence obtained from changes in reversal potential (\(V_{rev}\)) was Cs\(^+\) (0.87) ≈ Na\(^+\) (1.0) ≈ Rb\(^+\) (1.01) ≈ K\(^+\) (1.11). The permeability for divalent cations was explored next with a pipette solution containing 75 mM CaCl\(_2\) or 75 mM MgCl\(_2\). Under these experimental conditions, no inward currents were detected, therefore suggesting that extracellular divalent cations do not significantly permeate this NSCC. Alternatively, these results might be compatible with divalent cation blockade of the channel. Furthermore, these results confirm that the NSCC is impermeable to anions.

\(P_o\) and conductance modulation by internal Ca\(^{2+}\). Figure 2A shows representative single-channel current recordings from an excised inside-out membrane patch (\(V_m\) = 60 mV) in symmetrical 145 mM sodium glutamate solution exposed to different [Ca\(^{2+}\)]. \(P_o\) increased in a Ca\(^{2+}\)-dependent manner approaching 1 at 2.6 mM [Ca\(^{2+}\)], allowing the estimation of the total number of channels present in the membrane patch. These recordings were used to construct the [Ca\(^{2+}\)]-to-\(P_o\) relationship depicted in Fig. 2B. A Hill function was fitted to the data with \(n = 1.54 ± 0.15\) and \(E_{50} = 435 ± 33\) \(\mu\)M. The single-channel recording at the bottom of Fig. 2A shows that, at higher [Ca\(^{2+}\)], unitary current amplitudes were consistently smaller. Therefore, the effect of [Ca\(^{2+}\)] on single-channel

Fig. 3. Effect of ATP on NSCC activity. A: single-channel activity recorded from a representative inside-out patch containing \(N = 3\) channels exposed to different intracellular ATP concentrations ([ATP]) in the presence of 2.6 mM [Ca\(^{2+}\)]. From top to bottom: 0, 50, and 500 \(\mu\)M [ATP]. B: Hill plot of the effect of ATP. Each membrane patch was exposed to all ATP concentrations (\(n = 5\)). Continuous line represents Hill equation fitted to the data with \(n = 0.77 ± 0.08\) and \(E_{50} = 32 ± 4\) \(\mu\)M. Data are expressed as means ± SE. Error bars are shown if bigger than symbols.

Fig. 4. Hydroxyl radical (\(\mathrm{OH}\)) modulates NSCC activity. A: single-channel activity recorded from an inside-out patch containing \(N = 6\) active channels. Control recording was obtained in a sodium glutamate internal solution containing 300 \(\mu\)M free Ca\(^{2+}\) concentration. The intracellular face of the patch was then exposed to 5 mM H\(_2\)O\(_2\) and 5 \(\mu\)M Fe\(_2\)SO\(_4\) resulting in increased channel activity. After 4 min of washing, 1 mM DTT was applied. At right of each trace, amplitude histograms fitted to a binomial function are shown. These histograms were used for \(P_o\) calculations as described in MATERIALS AND METHODS. B: \(P_o\)-vs.-time plot for the tracing shown in A. \(V_m\) was kept constant at -60 mV and [Ca\(^{2+}\)] at 300 \(\mu\)M (●) and in the presence of 5 mM H\(_2\)O\(_2\) plus 5 \(\mu\)M Fe\(_2\)SO\(_4\) (○). \(P_o\) was measured every 30 s to test for gating stability of the channels. C: summary of results (3-5 patches) showing the effect of H\(_2\)O\(_2\) and Fe\(_2\)SO\(_4\) and its reversion by DTT. Data are means ± SE. *\(p < 0.05\) with respect to control.
Conductance (γ) was explored next. As shown in Fig. 2C, increasing [Ca\(^{2+}\)], significantly decreased single-channel conductance. A Michaelis-Menten function was fitted to the data with maximal γ\(_{\text{max}}\) = 23.6 ± 0.6 pS and K\(_d\) = 10.1 ± 0.8 mM, suggesting that Ca\(^{2+}\) from the intracellular compartment blocks the channel.

P\(_o\) is modulated by internal ATP. Most of the NSCC reported so far are inhibited by intracellular adenine nucleotides. Figure 3A shows representative single-channel current recordings from an excised inside-out membrane patch in symmetrical 145 mM sodium glutamate and 2.6 mM Ca\(^{2+}\) in the presence of intracellular ATP. On addition of increasing concentrations of the nucleotide, NSCC activity decreased without changes in unitary conductance. Figure 3B summarizes the effect of internal ATP on the NSCC activity. A Hill function was fitted to the data with n = 0.77 ± 0.08 and IC\(_{50}\) = 32 ± 4 μM. ADP was equally effective in decreasing the activity of NSCC (not shown).

Effect of ·OH and SH residue reducing agents on NSCC activity. The effect of ·OH on the NSCC activity was explored by exposing the cytoplasmic side of the membrane patch to 5 mM H\(_2\)O\(_2\) and 5 μM Fe\(^{2+}\) to produce ·OH in situ. Figure 4A shows representative single-channel recordings at 300 μM [Ca\(^{2+}\)] and their respective amplitude histograms fitted to a binomial function. Figure 4B shows the stability plot for the recordings depicted in Fig. 4A. In the absence of H\(_2\)O\(_2\) and Fe\(^{2+}\), P\(_o\) was 0.32 ± 0.03 (n = 5). After 2 min in the presence of H\(_2\)O\(_2\) and Fe\(^{2+}\), P\(_o\) increased to 0.44 ± 0.02 (n = 5, P < 0.05). The increase in P\(_o\) could not be reversed by extensive washing with an oxidant-free solution (10–15 min). However, exposure of the intracellular side to the SH reducing agent DTT (1 mM) after application of H\(_2\)O\(_2\) and Fe\(^{2+}\) completely reversed the increase in P\(_o\) (n = 3, Fig. 4A and C).

To rule out the possibility that H\(_2\)O\(_2\) could be directly affecting channel activity, we added 5 mM H\(_2\)O\(_2\) and 100 μM desferrioxamine to the sodium glutamate, 300 μM Ca\(^{2+}\) bath solution. If applied before H\(_2\)O\(_2\) addition, desferrioxamine prevents ·OH formation via the Fenton reaction by chelating any traces of heavy metals present in the solution. Under these experimental conditions, P\(_o\) values were 0.36 ± 0.02 (control), 0.34 ± 0.02 (desferrioxamine), and 0.35 ± 0.02 (desferrioxamine plus H\(_2\)O\(_2\)) (n = 6, not statistically different), showing that H\(_2\)O\(_2\) and desferrioxamine per se do not modify NSCC activity (Fig. 5A). Moreover, Fe\(^{2+}\) by itself (10–100 μM) did not modify P\(_o\) (n = 5; P\(_o\)-control: 0.37 ± 0.04, P\(_o\)-Fe\(^{2+}\): 0.37 ± 0.03). To confirm that ·OH specifically were responsible for the observed increase in P\(_o\), the ·OH scavenger MCI-186 was used. As depicted in Fig. 5B, addition of 0.5 mM MCI-186 to the bath solution prevented the increase in P\(_o\) triggered by H\(_2\)O\(_2\) plus Fe\(^{2+}\) (n = 6). None of the reagents (desferrioxamine, Fe\(^{2+}\), H\(_2\)O\(_2\), MCI-186, and DTT), alone or combined, affected NSCC unitary conductance.

Modification of SH groups by DTNB. ·OH are nonspecific oxidizing molecules. To determine whether SH residues were involved in NSCC modulation by oxidizing agents, we used 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB). This hydrophilic oxidative reagent specifically targets SH groups in proteins in a reaction that involves a thiol-disulfide exchange mechanism, pointing, therefore, to cysteine residues. Figure 6A shows...
representative single-channel recordings of the effect of internally applied DTNB on channel activity and their respective amplitude histograms fitted to a binomial function. Figure 6B shows the stability plot for the recordings depicted in Fig. 6A. On addition of 0.5 mM DTNB, $P_0$ increased from 0.36 ± 0.01 to 0.47 ± 0.02 ($n = 5$, $P < 0.05$), without changes in unitary conductance. Control $P_0$ values were not restored by withdrawal of DTNB from the cytoplasmic face of the patch membrane, suggesting a covalent modification. However, DTNB-induced $P_0$ increase was fully reverted by application of 1 mM DTT (Fig. 6, A and C, $n = 3$). These results indicate that the effect of DTNB is specifically related to oxidation of SH groups.

**Effect of GSSG on NSCC activity.** In physiological conditions, the GSH/GSSG ratio plays a critical role in maintaining intracellular redox balance. Normally, glutathione redox status ([GSH]:[GSSG]) greatly favors GSH. However, during oxidative stress, GSSG accumulates, shifting the redox balance to an oxidizing state (1). To determine whether a physiological oxidizing molecule could modulate NSCC activity, excised inside-out membrane patches were exposed to 2 mM GSSG. Figure 7A depicts a representative experiment before and after addition of GSSG and the respective amplitude histograms fitted to a binomial function. Figure 7B shows the stability plot for the recordings depicted in Fig. 7A. On GSSG addition, $P_0$ increased from 0.34 ± 0.02 to 0.52 ± 0.05 ($n = 7$, $P < 0.05$), without changes in unitary conductance. $P_0$ did not return to control values after GSSG removal. However, as shown in Fig. 7, A and C, application of 2 mM GSH as a reducing agent restored $P_0$ to control values (0.38 ± 0.03, $n = 3$).

**Effect of GSSG on NSCC $\frac{[\text{Ca}^{2+}]}{[\text{GSSG}]}$-to-$P_0$ relationship.** To explore whether the $\text{Ca}^{2+}$ sensitivity at more physiological or pathophysiological $[\text{Ca}^{2+}]$ of the NSCC is affected by oxidation, experiments were performed using the physiological oxidizing agent GSSG. Excised inside-out patches were exposed to 2 mM GSSG and thereafter to different $[\text{Ca}^{2+}]$. Figure 8C, continuous line, $n = 6–10$ for each $[\text{Ca}^{2+}]$ depicts the result of such experiments. Compared with nonoxidized channels (dashed line taken from Fig. 2B), oxidized channels show an enhanced sensitivity to $\text{Ca}^{2+}$. Channel openings could be consistently observed at low micromolar $[\text{Ca}^{2+}]$, compared with nontreated channels in which no openings were detected over a 30-min observation period.

**DISCUSSION**

$\text{H}_2\text{O}_2$ is a biologically active oxygen-derived intermediate compound that plays a major role as an intracellular signaling molecule (43). However, $\text{H}_2\text{O}_2$ is also associated with a series of alterations in different types of cells that may lead to cell damage and cell death (10, 50). Necrosis is accompanied by an increase in cell volume in part because of osmotically driven water fluxes. Several studies have shown that $\text{Na}^+$ permeability is augmented in various cell types under oxidative stress (5, 37, 44). In the present study, we have characterized a ~20 pS ATP and fenamate-sensitive $\text{Ca}^{2+}$-activated NSCC in HTC cells that is activated by oxidizing agents applied to the intracellular aspect of the membrane patch. This NSCC is activated by oxidizing agents applied to the intracellular aspect of the membrane patch.
almost equally permeable to Na⁺, K⁺, Rb⁺, and Cs⁺ and impermeant to Ca²⁺. Channel activity is dependent on [Ca²⁺], and, as judged from the analysis of the data, at least two Ca²⁺-ions are required to activate the channel. The half-maximal activation concentration in excised membrane patches is ~450 μM. Although this [Ca²⁺], is infrequently found in normal cells, damaged cells, however, exhibit significant intracellular Ca²⁺ overload, which is linked to cell death (39). Interestingly, GSSG-oxidized channels exhibit a leftward shift in the [Ca²⁺]-Pₒ relationship, indicating that under oxidizing intracellular conditions less Ca²⁺ is required to activate these channels. Moreover, it is possible to observe a discrete but consistent channel activity at high nanomolar Ca²⁺ concentrations in GSSG-oxidized channels. These observations suggest that these NSCCs can be activated by Ca²⁺ concentrations closer to the relevant physiological and pathophysiological [Ca²⁺] range.

In agreement with data collected from several NSCC (12, 22, 47), the Ca²⁺-activated NSCCs in HTC cells were efficiently blocked by adenine nucleotides. Because of its low oxidizing potential, usually H₂O₂ is not by itself reactive enough with organic molecules (4). Nevertheless, H₂O₂ has the ability to generate highly reactive ·OH through its interaction with redox-active transitional metals (3). ·OH result from the breakdown of H₂O₂ via the Fenton reaction and by interaction of superoxide with H₂O₂ through the Haber-Weiss reaction. Highly reactive ·OH readily reacts with a variety of molecules, such as amino acids and lipids, by removing hydrogen or by addition to unsaturated bonds.

Here we describe that applying H₂O₂ simultaneously with Fe²⁺ to the cytoplasmic face of excised inside-out membrane patches of HTC cells containing Ca²⁺-activated NSCCs produces a significant increase in Pₒ, without affecting single-channel conductance. Although we cannot exclude the possibility that these conditions may affect other proteins or components associated with the membrane patch, it can be assumed on the basis of previous work in channels in reconstituted systems (34, 46) that the primary target of ·OH is the channel-forming protein. The effect of Pₒ increase could be ascribed to the generation and oxidizing action of ·OH, as demonstrated by the experiments in which desferrioxamine and MCI-186 were used. The increase in Pₒ was not reversed by washing, although Pₒ returned to normal values after the addition of the reducing agent DTT. This observation indicates that ·OH could be targeting some exposed amino acid residues. To address whether the ·OH-induced Pₒ increase of NSCC could be attributed to the oxidation of free SH residues of cysteine, membrane patches were exposed to DTNB, a hydrophilic agent that reacts specifically with free SH groups in proteins forming disulfide bonds. In the presence of 0.5 mM DTNB, Pₒ value increased to an extent similar to that observed with H₂O₂ and Fe²⁺, an effect fully reversed by addition of 1 mM DTT. These results indicate that NSCC are modulated by agents that modify the redox condition of SH groups, a result similar to that reported for Ca²⁺-activated K⁺ channels (8, 46). The involvement of cysteinyl residues in the modulation of Pₒ was investigated further by exposing the NSCC to GSSG. Addition of 2 mM GSSG resulted in a significant increase in Pₒ to values similar to those observed with H₂O₂-Fe²⁺ or DTNB. The effect of GSSG was completely reversed by its reducing counterpart, GSH, confirming that SH groups of cysteine residues are responsible for the change in the gating behavior of NSCC in HTC cells. Similar results were observed in calf pulmonary artery endothelial cells, in which the activation of a NSCC by tert-butylhydroperoxide was mimicked by GSSG and reversed by GSH (30). Effects of SH modification on channel gating, but not on permeation, have also been reported for cloned K⁺, Na⁺, and Ca²⁺ channels (13, 26, 46). The results presented here are thus compatible with the existence of SH groups susceptible to redox modulation on the cytosolic side of NSCC in HTC cells. In summary, we have extended our previous findings on activation of NSCCs in liver cells by free-radical donors (5) by identifying ·OH as a relevant agent participating in this process. Under pathological conditions associated with oxidative stress, intracellular Ca²⁺ increase, and ATP depletion, NSCC would become activated, generating the influx of cations that results in necrotic cell volume increase. Moreover, the results presented here strongly suggest that ·OH-induced NSCC activation is mediated by specific modifications of cysteine residues, presumably located on the intracellular aspect of the channel protein.

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