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Hydrogen peroxide induced responses of cat tracheal smooth muscle cells

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1 The effects of hydrogen peroxide (H_2O_2) (10^{-6}–10^{-3} M) on membrane potential, membrane currents, intracellular calcium concentration, resting muscle tone and contractions elicited by electrical field stimulation (EFS) and carbachol were examined in cat tracheal strips and isolated smooth muscle cells.

2 H_2O_2 (10^{-4} and 10^{-5} M) enhanced the amplitude of contractions and excitatory junction potentials (e.j.p.) evoked by EFS without changing muscle tone and resting membrane potential of the tracheal smooth muscle, and enhanced the contraction induced by carbachol (10^{-8} M). At an increased concentration (10^{-3} M), H_2O_2 elevated resting muscle tone and marginally hyperpolarized the membrane in the majority of the cells.

3 In 51 out of 56 cells examined, H_2O_2 (10^{-6}–10^{-3} M) elicited an outward current at a holding potential of −40 mV and enhanced the frequency of the spontaneous transient outward current (STOC). In 20 cells the outward current was preceded by a small inward current. In the other cells, H_2O_2 elicited only an inward current or did not affect the background current.

4 In Ca^{2+} free solution the action of H_2O_2 on the resting muscle tone, STOCs, background current and on the current induced by ramp depolarization was significantly reduced.

5 H_2O_2 (10^{-4} M) increased the intracellular ionized calcium concentration both in the absence and presence of external Ca^{2+}. However, the effect developed faster and was of a higher amplitude in the presence of external Ca^{2+}.

6 These results suggest that H_2O_2 increases intracellular Ca^{2+}, with a subsequent augmentation of stimulation-evoked contractions, and enhances Ca^{2+} and voltage-sensitive potassium conductance.

Keywords: Cat trachea; hydrogen peroxide; patch clamp; potassium current

Introduction

Activation of alveolar macrophages, neutrophils, eosinophils and monocytes in the lung is generally considered to release highly reactive oxygen species (Babior *et al*., 1973; Drath & Karnovsky, 1975; De Chatelet *et al*., 1977; Blake *et al*., 1987). These reactive oxygen metabolites include superoxide anion radical (O_2^-), hydroxyl radical (OH), hypochlorite (HOCl) and hydrogen peroxide (H_2O_2), which are the mediators of cell and tissue injury during inflammatory processes (Blake *et al*., 1987; Sinclair *et al*., 1990; Bast *et al*., 1991; Kato *et al*., 1991).

It has been repeatedly suggested that the generation of reactive oxygen species in acute airway injury, inflammation and hyperoxia could induce airway hyperreactivity (Johnson *et al*., 1981; Rhoden & Barnes, 1989; Szarek, 1989; Szarek & Schmidt, 1990; Katsumata *et al*., 1990; Kato *et al*., 1991; Bast *et al*., 1991; Owen *et al*., 1991; Misawa & Arai, 1993a,b; Takahashi *et al*., 1993; Mátyás & Bauer, 1995; Rabe *et al*., 1995). Of the reactive oxygen species, H_2O_2 is the only living substance. It is membrane permeable and may therefore diffuse over considerable distances from its site of generation, causing cell damage at distant target sites (Blake *et al*., 1987), either itself or via its transformation to other even more deleterious short living reactive oxygen species (e.g. OH).

The effects of H_2O_2 on airway smooth muscle tone have been studied in several animal species including man, and H_2O_2 was found to contract bovine (Stewart *et al*., 1981), dog (Stewart *et al*., 1981; Gao & Vanhoutte, 1991), rabbit (Gupta & Prasad, 1992), guinea-pig (Rhoden & Barnes, 1989; Gupta & Prasad, 1992; Gao & Vanhoutte, 1993; Misawa & Arai, 1993a; Mátyás & Bauer, 1995), rat (Szarek & Schmidt, 1990), ferret (Morikawa *et al*., 1991) and human (Rabe *et al*., 1995) tracheal and bronchial muscle strips. Further, the effects of H_2O_2 are generally considered to be modulated by cyclo-oxygenase inhibitors or epithelium removal (Rhoden & Barnes, 1989; Stewart *et al*., 1981; Szarek & Schmidt, 1990; Gao & Vanhoutte, 1991; Hulsmann *et al*., 1994; Mátyás & Bauer, 1995; Rabe *et al*., 1995).

However, these experiments were mainly carried out by means of the tension recording method in tracheal or bronchial muscle strips and the details of actions of H_2O_2 on cat airways and airway smooth muscle cells have not been studied. We therefore investigated the effects of H_2O_2: (i) on resting muscle tone and contractions elicited by electrical field stimulation (EFS) or carbachol, (ii) on resting membrane potential and excitatory junction potentials (e.j.p.), (iii) on membrane currents and (iv) on intracellular free calcium concentration, by means of tension recording, microelectrode, patch clamp and intracellular calcium recording methods in cat tracheal strips or isolated single smooth muscle cells.

Methods

Tissue preparations

Adult mongrel cats of either sex (2–3 kg) were anaesthetized with sodium pentobarbitone (30–40 mg kg^{-1}, i.p.) and then bled. The trachea was removed and placed in aerated (97% O_2 and 3% CO_2) modified physiological salt solution (PSS) of the following ionic composition (mM): Na^+ 137.4, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, Cl^- 134, $H_2PO_4^-$ 1.2, HCO_3^- 15.5 and glucose 11.5 (pH 7.3–7.4). The ionic concentration of the nominally calcium free solution was (mM): Na^+ 140, K^+ 5, Mg^{2+} 1.2, Cl^- 147.4, HEPES 10 and glucose 10.0, the pH was adjusted with NaOH to 7.2.

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Mechanical recording

The tracheae were dissected free of the connective tissue and vasculature. Dorsal strips of transversally running tracheal smooth muscle were separated from the cartilage and the mucosa was removed leaving only the smooth muscle. The tracheal smooth muscle was cut to a width of 2–3 mm and length of about 5 mm and the preparations were bathed in aerated PSS.

For measurement of mechanical responses, the tracheal tissue was mounted vertically in a 1 ml organ bath through which the test solution, at 35–36°C, flowed continuously at a rate of 3 ml min^{-1}. One end of the strips was tied by a fine silk thread to a mechanotransducer (RCA-5734, Nihon Kohden) and the other to a hook at the bottom of the bath. The strips were set up with an initial tension of 0.1–0.2 g and mechanical activity was recorded in PSS with a pen recorder. EFS (10, 20, 30 pulses of 50 μs duration and 20–30 V strength at 20 Hz) was applied through a pair of Ag-AgCl plates fixed to the upper and lower sides of the inner surface of the chamber, so that the current would pass transversely across the tissue.

Membrane potential recording

For intracellular recording of resting membrane potential and e.j.ps, a conventional glass microelectrode filled with KCl (3 M) of 30–50 MΩ resistance was inserted into a cell from the outer surface of the tissue. The chamber in which the strips were mounted had a volume of 2 ml and was superfused at a rate of 3 ml min^{-1} with aerated PSS at 35–36°C. EFS (1–3 pulses of 50 μs duration and 20–30 V strength at 20 Hz) was applied to the nerves in the tissue through a pair of Ag-AgCl wires (3–5 mm apart) placed so that a current pulse would pass transversely across the tissue to induce e.j.p. To avoid recording artefacts due to twitch-like contractions, the preparation was pinned to the rubber plate in the chamber by use of insect pins with 100 μm diameter.

Patch clamp recording

The tracheal tube was cut into pieces containing two cartilaginous rings and was kept in the aerated PSS at room temperature for 60–90 min to allow the trachealis to relax fully. Three such pieces were transferred into 3 ml of nominally calcium-free dissociation solution, to which collagenase (2 mg ml^{-1}), pronase (0.1 mg ml^{-1}) and (−)-1,4-dithio-L-threitol (L-DTT, 1 mM) were added. The tissues were then maintained in this solution in a shaking water bath at 36°C for 32–36 min. After enzymatic digestion, the tissues were transferred to the calcium-free solution containing trypsin inhibitor (1 mg ml^{-1}) and kept at 4°C for 60 min. The epithelium was then removed, the trachealis was dissected free from the cartilage rings and cut into small pieces (1 × 1 mm). These were gently triturated to liberate individual myocytes and then stored in ice-cold PSS with 0.5 mM $CaCl_2$. In the actual experiments the cells were allowed to settle and adhere to the bottom of the recording chamber (0.3 ml). The rate of flow of the PSS solution was approximately 1.5 ml min^{-1}. The patch clamp experiments were performed at room temperature within 8 h after the dissociation of cells. Membrane currents were recorded with glass pipettes in the tight-seal whole-cell recording (Hamill *et al*., 1981) using the amphotericin perforated patch configuration (Rae *et al*., 1991). Heat polished patch pipettes were filled with artificial internal solution (IS) of the following ionic concentration (mM): K^+ 140, Ca^{2+} 0.4, Mg^{2+} 1, Cl^- 142.8, HEPES 20, Tris-EGTA 1, the pH was adjusted to 7.2 with KOH. This solution contained amphotericin B at a final concentration of 0.24 mg ml^{-1}. The pipette

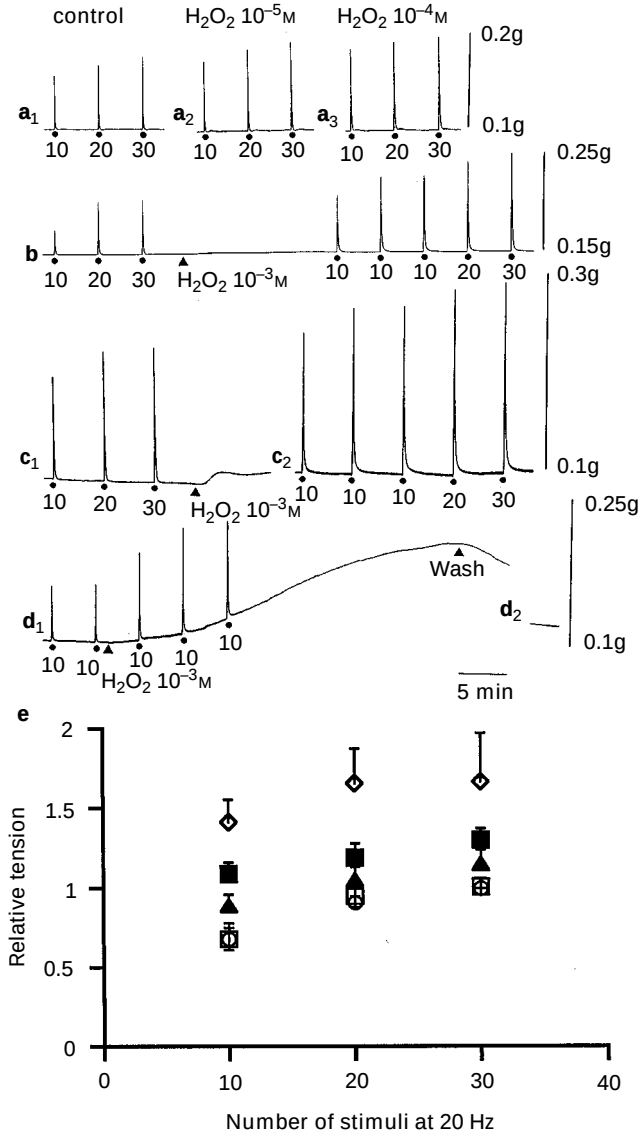

Figure 1 Effects of H_2O_2 on resting muscle tone and EFS-induced contractions of the cat isolated trachea. (a,b,c,d) Records from different muscle strips. Repetitive field stimulation of 10, 20, 30 pulses at 20 Hz (●) were applied. Time lags between a_1, a_2 and a_3, c_1 and c_2, d_1 and d_2 were 15, 10 and 20 min, respectively. (e) Relationship between number of pulses at 20 Hz and relative amplitude of contraction in the absence (○) and presence of H_2O_2 ((□) 10^{-6}, (▲) 10^{-5}, (■) 10^{-4}, (◇) 10^{-3} M). Amplitude of contraction evoked by 30 pulses in the absence of H_2O_2 was taken as a relative amplitude of 1.0. Results are expressed as mean, $n = 8$; vertical lines show s.e.mean.

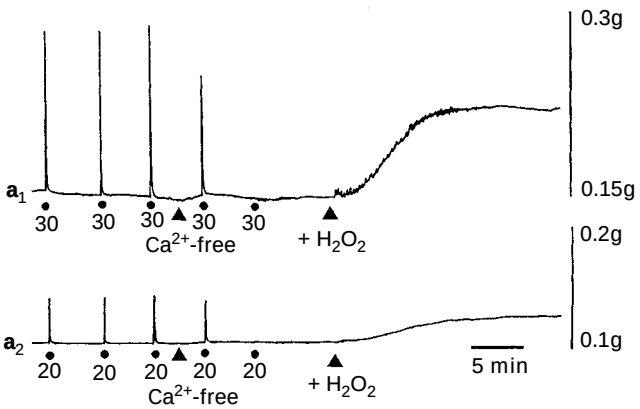

Figure 2 Effects of Ca^{2+} free (0.25 mM EGTA) solution on responses of the cat isolated trachea elicited by EFS (●, a_1 30 pulses, a_2 20 pulses at 20 Hz) and H_2O_2 (5×10^{-3} M). The triangles indicate introduction of the Ca^{2+} free solution and administration of H_2O_2.

had a tip resistance of 3–7 MΩ. Currents were recorded with an Axopatch-1 D amplifier interfaced to an IBM compatible computer by use of the software pClamp version 5.5 (Axon Instruments). The junction potential between pipette and bath solution was nulled before seal formation. Cell capacitance and series resistance were compensated electronically and acquired current signals were low-pass filtered at 1 kHz. To study the H_2O_2-induced current, in most of cells tested voltage-ramp commands were used to construct voltage-current relationships.

Measurement of intracellular Ca^{2+} concentration

Enzymatically dispersed cells were loaded with 2 μM of the acetoxymethylester of Ca^{2+} fluorescent dye fura-2 dissolved in PSS for 20 min at room temperature and thereafter for a further 20 min at 37°C. Fura-2 loaded cells were then transferred to a chamber of 0.5 ml volume which was mounted on an inverted-microscope (Diaphot TMD with special optics for epifluorescence, Nikon). All experiments were carried out at room temperature. In some experiments fura-2 (50 μM) was dialysed directly into the cell clamped at −40 mV through a patch pipette containing IS. The smooth muscle cells were excited with two alternative excitation wavelengths, 340 and 380 nm, (each slit 5 nm) applied by a spectrometer (Spex, Edison, N.J.). The obtained fluorescent intensities (F_{340}/F_{380}, respectively) were analysed by use of a customized software provided by Spex (DM-3000CM). The ratio of fura-2 fluorescence intensities excited by two u.v. lights, F_{340}/F_{380}, was calculated after subtraction of background fluorescence. After each experiment in PSS, the background fluorescence was taken from a cell free area just beside the cell examined. It was less than 10% of the fura-2 signal at either excitation wavelength. Because precise *in situ* calibration of $[Ca^{2+}]_i$ was difficult to perform, we used the fluorescent ratio F_{340}/F_{380}, as an index of $[Ca^{2+}]_i$.

Statistics and drugs

The summarized data are shown as mean values with s.e. Statistical differences were tested by Student's *t* test. The following drugs were used: propranolol, indomethacin and fura-2/AM (Wako Pure Chemical Industries), collagenase (type I), (−)-1,4-dithio-L-threitol, trypsin inhibitor (type I-S) and pronase (Sigma), amphotericin B (Fungizone, Life Technologies).

Results

Effects of H_2O_2 on mechanical responses

In the cat trachea, EFS produced a phasic contraction with constant amplitude in the presence of indomethacin (10^{-5} M) and propranolol (10^{-6} M), which is sensitive to atropine and tetrodotoxin (Jing *et al*., 1995; Takahashi *et al*., 1995). When the number of pulses was increased in a stepwise manner at a constant stimulus intensity and frequency (20 Hz), the amplitude of the contractions increased proportionally. Under these conditions, H_2O_2 (10^{-5} M) enhanced the amplitude of EFS-induced contractions concentration-dependently (Figure 1a–d, e). H_2O_2 (10^{-5} and 10^{-4} M) did not affect the muscle tone significantly, although at a higher concentration (10^{-3} M) it

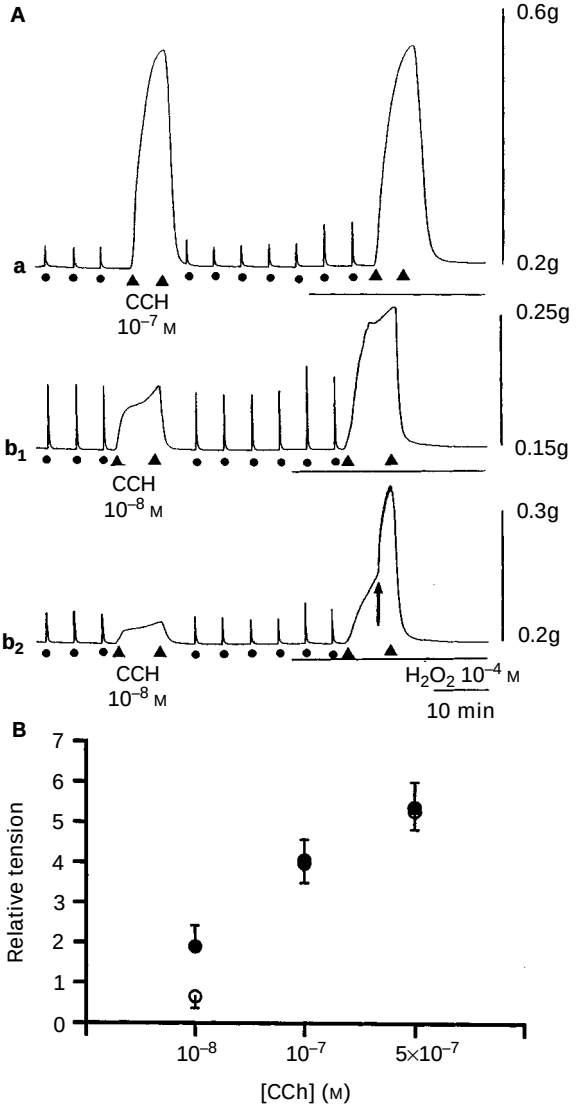

Figure 3 Effects of H_2O_2 on responses of the cat isolated trachea elicited by EFS (●, 10 pulses at 20 Hz) and carbachol (▲, CCh, $10^{-8}-5\times10^{-7}$ M). The second triangle after each application of CCh indicates the washing and the arrow in (b_2) indicates the phase of fused spontaneous rhythmic contractions. (A) Original records from three different muscle strips; (B) relationship between concentration of carbachol and relative amplitude of contraction of the tracheal strips induced by carbachol in the presence (●) and absence (○) of H_2O_2. The horizontal lines indicate the duration of the presence of H_2O_2 in the bathing fluid. Results are expressed as mean, $n=8-12$; vertical lines show s.e.mean.

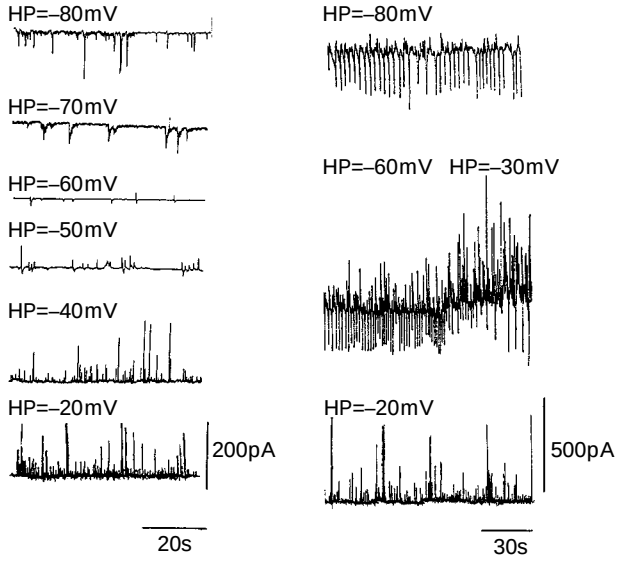

Figure 4 Cat tracheal smooth muscle cells exhibit spontaneous membrane currents. Spontaneous transient outward (STOC) and inward currents (STIC) are shown from two different cat tracheal smooth muscle cells. The STOCs were mainly apparent at potentials more positive than −60 mV and STICs at potentials more negative.

produced a slow and long lasting increase in muscle tone with or without initial phasic contractions (Figure 1b–c). After treatment of the tissue with Ca^{2+} free (0.25 mM EGTA) solution for 20 min, the contractions induced by EFS (20 and 30 pulses at 20 Hz) disappeared, H_2O_2 in concentrations up to 10^{-3} M did not increase significantly the muscle tone ($n=8$). However, at concentrations of 5×10^{-3} M ($n=12$, Figure 2) and 10^{-2} M ($n=7$, not shown), H_2O_2 evoked a gradual increase in muscle tone, indicating that H_2O_2 in high concentrations

Table 1 Membrane currents induced by H_2O_2 in cat tracheal smooth muscle cells

Conc. of H_2O_2	*Inward* (PA)	*Outward* (PA)	n
10^{-6} M	0.0±0.0	15.0±4.1	6
10^{-5} M	2.1±1.5	42.9±19.5	7
10^{-4} M	36.6±10.5	145.0±36.1	22
10^{-3} M	24.5±9.9	188.0±47.0	21

Data shown are means±s.d.

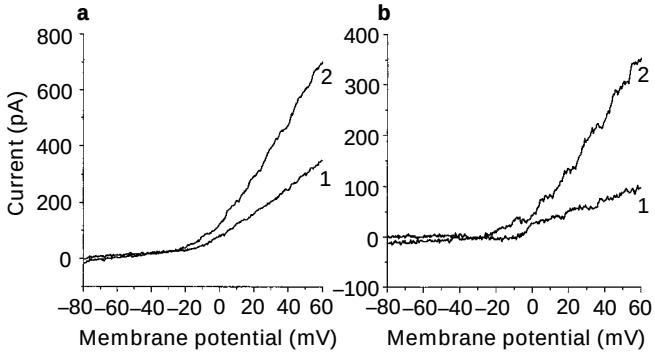

Figure 6 Current-voltage relationship of $I_{H_2O_2}$. Ramp depolarization (from −80 to +60 mV, with holding potential = −40 mV) elicited an outwardly rectifying current. This current was enhanced in the presence of H_2O_2, mainly in its outward component. The highest concentration of H_2O_2 slightly enhanced also its inward component. (a_1) Before, (a_2) in the presence of 10^{-3} M H_2O_2. (b) Difference between control response and the response in the presence of two concentrations of H_2O_2 (b_1, 10^{-4} M; b_2, 10^{-3} M) of the same cell.

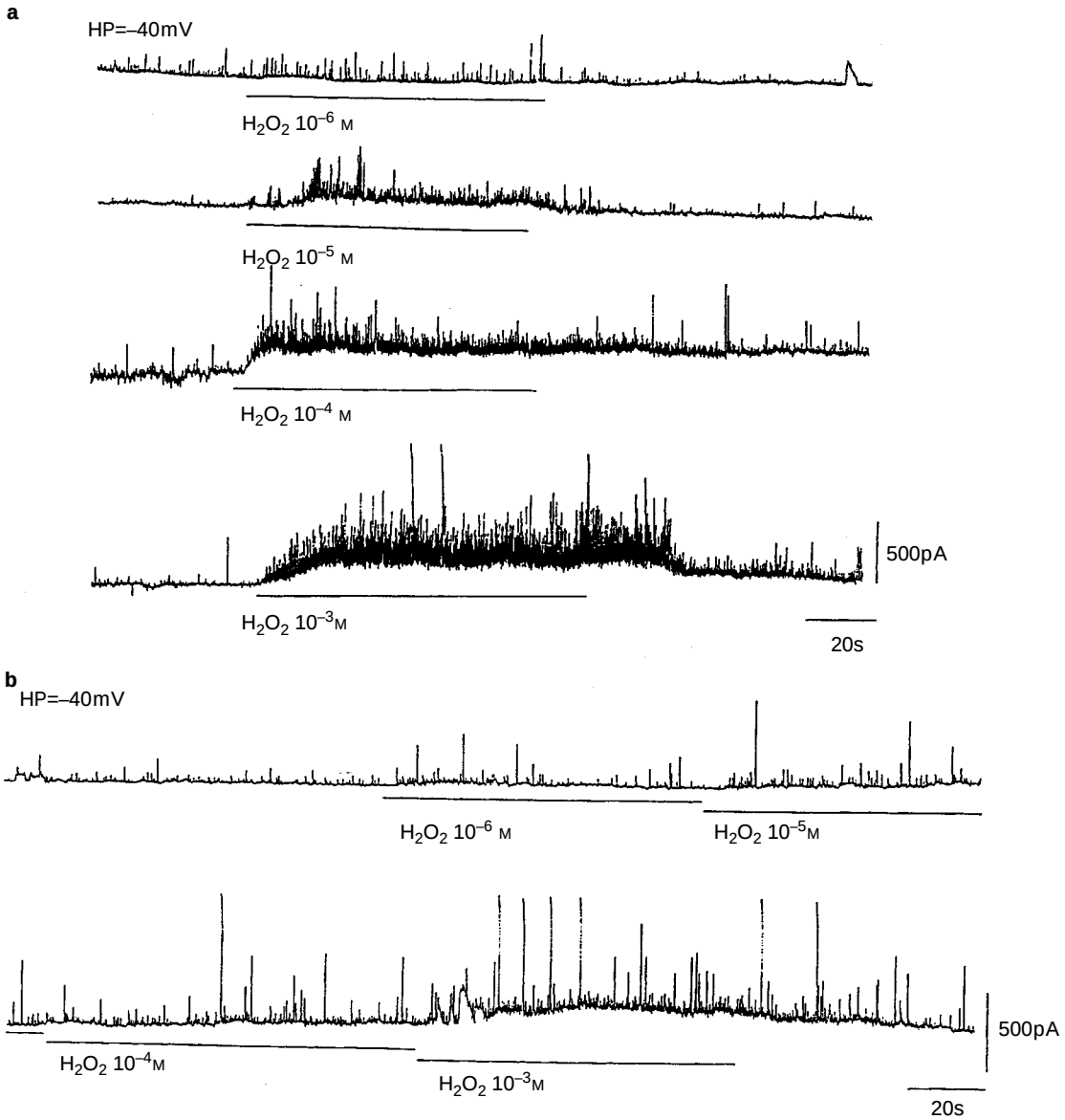

Figure 5 Enhancement of the amplitude and frequency of STOCs and outward currents evoked by H_2O_2 (10^{-6}–10^{-3} M) applied in a non-cumulative (a) and cumulative (b) manner in two different cat tracheal smooth muscle cells. The holding potential and duration of the presence of the given H_2O_2 concentration in the bathing fluid are indicated.

could directly release stored Ca^{2+} or Ca^{2+} bound to the membrane.

To investigate the mechanisms involved in the enhancement of EFS-induced contraction by H_2O_2, we observed the effects of H_2O_2 (10^{-4} M) on contractions induced by carbachol ($10^{-8}-5\times10^{-7}$ M). H_2O_2 enhanced the contractions evoked by a relatively low concentration (10^{-8} M) of carbachol, but did not significantly affect those induced by higher concentrations of carbachol (10^{-7} and 5×10^{-7} M) in the presence of indomethacin (10^{-5} M) (Figure 3A,B). In the presence of H_2O_2 (10^{-4} M) the initial phase of the carbachol (10^{-8} M)-induced contraction was in some preparations composed of rhythmic increments of the muscle tone (Figure $3Ab_2$).

Effects of H_2O_2 on resting membrane potential and e.j.p.

To study further the mechanisms involved in H_2O_2-induced enhancement of contractions evoked by EFS, we observed the effects of H_2O_2 on the resting membrane potential and e.j.ps of the tracheal smooth muscle cells in the presence of indomethacin (10^{-5} M). The mean value of the resting membrane potential of the tracheal smooth muscle cells was -63.6 ± 2.9 mV ($n=26$) and H_2O_2 (10^{-5} and 10^{-4} M) did not affect the resting membrane potential at all ($n=12$, paired t test). However, at an increased concentration (10^{-3} M), H_2O_2 marginally hyperpolarized the cell membrane from -64.5 ± 3.3 to -68.1 ± 2.9 mV ($n=14$, $P<0.05$). H_2O_2 (10^{-4} M) increased the relative amplitude of e.j.ps (control amplitude was taken as unity) to 1.31 ± 0.19 ($P<0.01$, $n=4$) without changing significantly the resting membrane potential. These results together with the contractile experiments suggest that H_2O_2 may enhance the EFS-induced contraction through pre- and postjunctional actions.

Effects of H_2O_2 on membrane currents

Enzymatically dispersed single cells from cat tracheal smooth muscle had a smooth and elongated appearance and adhered

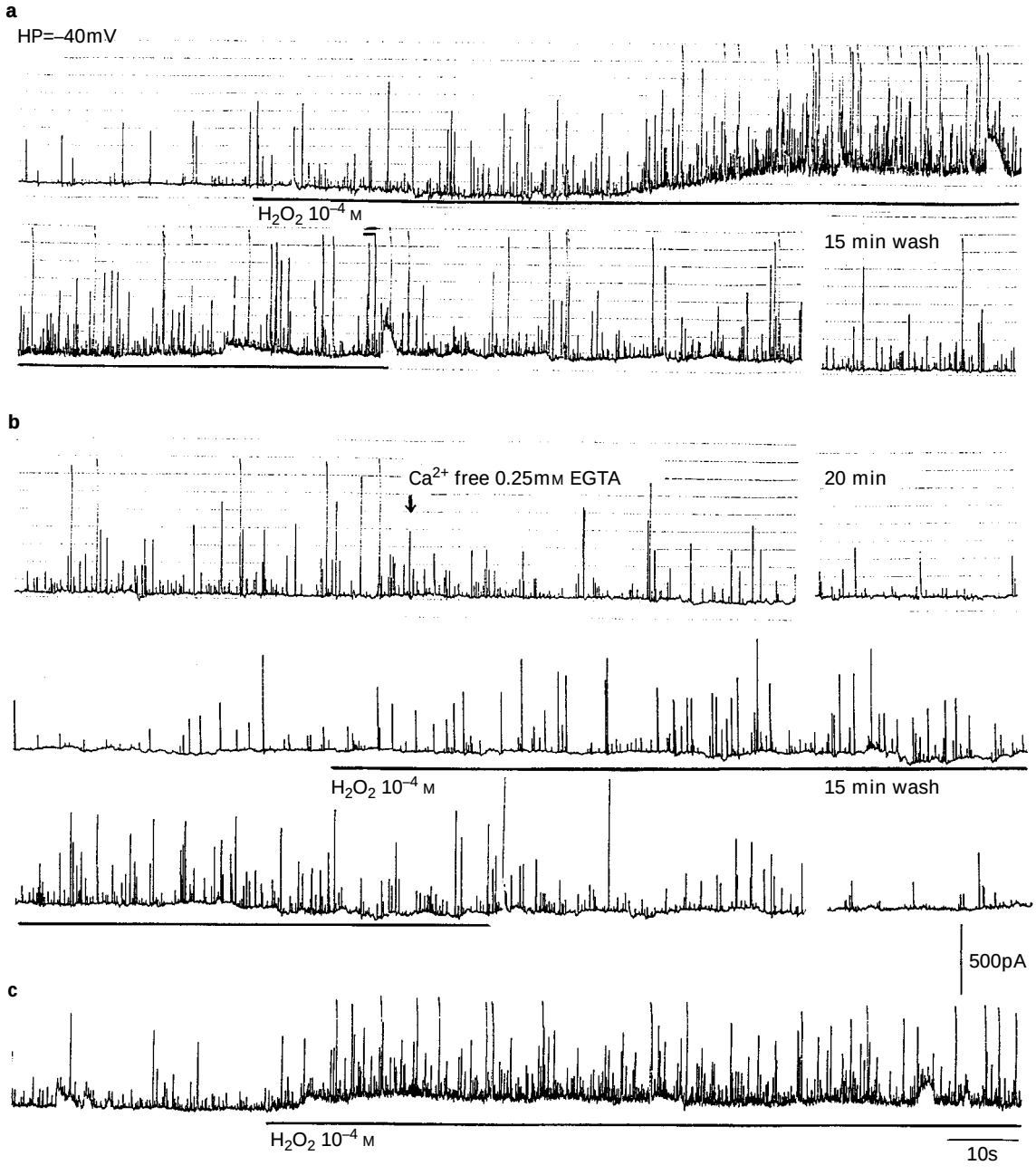

Figure 7 Effects of H_2O_2 in the presence (a) control response, (c) washout and absence (b) of 2.5 mM extracellular Ca^{2+} at a holding potential (HP) of -40 mV. The presence of H_2O_2 (10^{-4} M) in the bathing fluid is indicated by horizontal lines.

well to the bottom of the recording chamber. In our experiments only relaxed cells were used. Some of the cells contracted to H_2O_2 added to the bath, while others did not change their shape markedly. However, the length of the cells was not measured in parallel with the current recording.

In the tracheal smooth muscle cells spontaneous transient outward (STOC) or inward currents (STIC) could be recorded at a holding potential of -60 mV, which is close to the resting membrane potential of this muscle (see above). At more negative potentials the generation of STICs was more pronounced, while at more positive potentials STOCs became more apparent. In some cells both STICs and STOCs were generated simultaneously at a holding potential of -30 mV (Figure 4).

At -40 mV most of the voltage-clamped cells generated STOCs. Under these experimental conditions, H_2O_2 (10^{-6}–10^{-3} M) elicited an outward current in 31 of the 56 cells (Figure 5 and also see Table 1) with enhanced STOC activity. The slow outward current peaked within 15–30 s and lasted for over a minute, depending on the duration of the presence of H_2O_2 in the bathing fluid (Figure 5a, Figure 7). This effect of H_2O_2 was less pronounced with its cumulative than noncumulative application (Figure 5a and b). In 20 of the 56 cells, the outward current was preceded by a small inward current accompanied by increased STOC activity (e.g. Figure 7). The responses of the other 5 cells differed from those described above. In three of them, H_2O_2 elicited only an inward current with increased STOC activity, while in two cells it only enhanced the STOC activity without changing the background current. There was also one cell which generated a transient repetitive inward current during prolonged application of H_2O_2 with progressive reduction in its amplitude.

Table 1 shows a summary of the mean values of the membrane currents evoked by different concentrations of H_2O_2.

The current-voltage (I-V) relationship of the net membrane currents evaluated by ramp depolarizations (from -80 to $+60$ mV in 300 ms, with holding potential of -40 mV) was linear at potentials more negative than -20 mV and outwardly rectifying at more positive potentials (Figure 6a). H_2O_2 (10^{-4} and 10^{-3} M) increased the amplitude of the ramp currents mainly in its outward portion (at the potentials more positive than -25 mV; Figure 6a). Subtraction of the control trace from the one obtained in the presence of H_2O_2 gave a non-linear *I*-V relationship, which became steeper with higher concentrations of H_2O_2 (Figure 6b).

To investigate the role of extracellular Ca^{2+} in the action of H_2O_2 on membrane currents, experiments were carried out in calcium free PSS, in which Ca^{2+} was omitted and 0.25 mM EGTA was added. Due to the long lasting effects of H_2O_2 after washing out, it was difficult to observe the effects of repeated administration of H_2O_2 in most of the cells examined. Thus the cells were perfused either with calcium free EGTA containing PSS for 20 min before the first administration of H_2O_2 or following the application of H_2O_2 in Ca^{2+}-containing PSS. In calcium free PSS containing EGTA, the amplitude and frequency of STOCs (Figure 7b) and the amplitude of the outward current elicited by ramp depolarization was reduced (by 10–15%). The action of H_2O_2 on STOCs and outward current was less pronounced than in the presence of extracellular Ca^{2+}. At the holding potential of -40 mV, the degree of changes in the amplitude of H_2O_2 (10^{-4} and 10^{-3} M) induced outward current was only $6.4 \pm 5.7\%$ ($n=5$) and $10.2 \pm 6.3\%$ ($n=4$) of the controls, respectively. Moreover, H_2O_2 only marginally increased the STOC frequency in the absence of extracellular Ca^{2+}. However, when Ca^{2+} was re-introduced in the bathing solution, the action of H_2O_2 on STOCs was restored (Figure 7c).

Effects of H_2O_2 on intracellular calcium

The effects of H_2O_2 on membrane currents was dependent on $[Ca^{2+}]_0$, suggesting that the actions of H_2O_2 may be mediated by changes in $[Ca^{2+}]_i$. We therefore measured $[Ca^{2+}]_i$ of tracheal smooth muscle cells, with a fluorescent Ca indicator fura-2. Exposure of the cat tracheal smooth muscle cells to H_2O_2 (10^{-4} M) induced a gradual increase in $[Ca^{2+}]_i$ under both unclamped and clamped (-40 mV) conditions. This effect of H_2O_2 developed faster and achieved significantly higher amplitudes in the presence of extracellular calcium than in its absence (Figure 8).

Discussion

The present results can be summarized as follows: (i) in cat tracheal smooth muscle, relatively low concentrations of H_2O_2 (10^{-5}–10^{-4} M) enhanced the contractions evoked by EFS and carbachol and increased the amplitude of e.j.ps, without changing the resting muscle tone or resting membrane potential, (ii) at a concentration of 10^{-3} M, H_2O_2 elevated the muscle tone in the presence of extracellular Ca^{2+} and at higher concentrations (5×10^{-3} and 10^{-2} M) also in its absence, (iii) H_2O_2 (10^{-5}–10^{-3} M) induced outward currents and enhanced STOC activity, and in a concentration of 10^{-3} M it induced a slight membrane hyperpolarization in most cells, and (iv) H_2O_2 produced a slow sustained increase in $[Ca^{2+}]_i$.

Until now it was generally considered that H_2O_2-induced contractions observed in the guinea-pig tracheal or human bronchial smooth muscle strips were mediated, at least in part, by cyclo-oxygenase products. This was mainly substantiated by the finding that reactive oxygen species, including H_2O_2

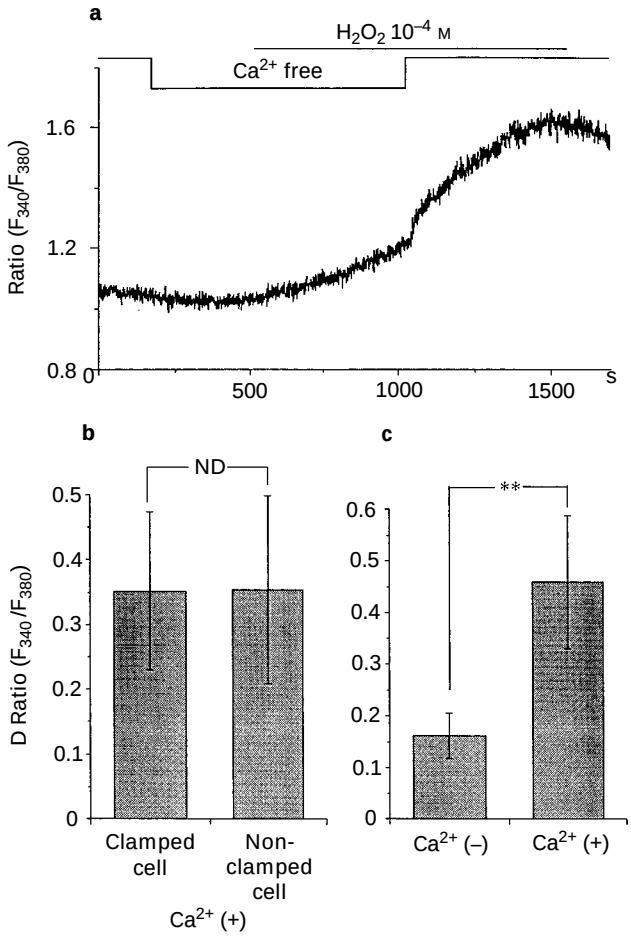

Figure 8 Fura-2 luminescence changes elicited by H_2O_2 (10^{-4} M). (a) Original record of H_2O_2 effects under Ca^{2+}-free conditions and after including Ca^{2+} into the solution. (b) Mean ($\pm$s.e.) luminescence in cells clamped at a holding potential of -40 mV ($n=4$) and non-clamped cells ($n=4$). (c) Luminescence elicited by H_2O_2 in the absence ($Ca^{2+}(-)$) and presence ($Ca^{2+}(+)$) of extracellular calcium in the bathing fluid ($n=7$). **Indicate statistically significant difference ($P<0.01$).

catalyse the oxidative modification of lipids and trigger the metabolism of arachidonic acid and release of its products (Harlan & Callahan, 1984; Sporn *et al*., 1988). Actually, indomethacin and flubiprofen shifted the concentration-response curve of the H_2O_2-induced contractions to the right and attenuated the maximal contractions (Rhoden & Barnes, 1989; Gao & Vanhoutte, 1993; Mátyás & Bauer, 1995; Rabe *et al*., 1995).

The present mechanical and membrane potential recordings with cat tracheal muscle strips were carried out in the presence of indomethacin in order to obtain stable contractions or e.j.ps (Ito, 1991; Jing *et al*., 1995; Takahashi *et al*., 1995), and yet H_2O_2 induced enhancements of muscle tone and of the amplitude of contractions and e.j.ps. Thus this H_2O_2 action could not have involved any cyclo-oxygenase products of arachidonic acid. This fact may also explain the discrepant efficacies of H_2O_2 to increase the muscle tone observed in previous studies as compared to the present one; 10^{-3} M was needed in the present study, while 10^{-6} or 10^{-5} M was sufficient in previously described experiments (Rhoden & Barnes, 1989; Gao & Vanhoutte, 1993; Rabe *et al*., 1995). The present results indicate that H_2O_2 can potentiate the contractile responses of the airway smooth muscle through mechanisms other than activation of the cyclo-oxygenase pathway of arachidonic acid.

On the other hand, our present mechanical recordings under Ca^{2+} free conditions and the experiments measuring $[Ca^{2+}]_i$ clearly showed that H_2O_2 was able to increase free $[Ca^{2+}]_i$, regardless the presence or absence of external Ca^{2+}. In the absence of external Ca^{2+}, H_2O_2 increased free $[Ca^{2+}]_i$ and muscle tone only slowly. However, a sudden increase was evoked on readdition of Ca^{2+} to the bathing solution, which was followed by a gradual increase in free $[Ca^{2+}]_i$. These observations are compatible with the idea that H_2O_2 may penetrate the muscle membrane, induce slow liberation of Ca^{2+} from internal stores and also cause a significant net Ca^{2+} influx through the plasma membrane. The precise mechanisim involved in these actions of H_2O_2 remains unknown. However, it is well documented that lipid or protein peroxidation caused by reactive oxygen species, including H_2O_2 decreases the membrane fluidity and disrupts the barrier functions of the membrane (see for example Blake *et al*., 1987), thereby increasing the membrane permeability for Ca^{2+}. Thus the sustained increase in the muscle tone, with or without initial phasic contractions evoked by H_2O_2 corresponds well with the observed gradual increase in free $[Ca^{2+}]_i$ induced by H_2O_2.

The increase in free $[Ca^{2+}]_i$ may also explain the increasing actions of H_2O_2 on STOC frequency and generation of outward currents. Janssen & Sims (1994) showed that spontaneous release of internally sequestered Ca^{2+} evoked transient currents, which were in some cases accompanied by rhythmic contractions. Several types of potassium currents were recorded from tracheal smooth muscle cells (Hisada *et al*., 1990; Muraki *et al*., 1990). Besides STOCs, or according to Hiasada's nomenclature the spontaneously oscillatory outward currents (I_O), transient outward current (I_T) and sustained outward current (I_S) were the major outward potassium currents recorded in the trachealis. Hisada *et al*. (1990) found that both I_O and I_T were sensitive while I_S was resistant to the blockade of Ca^{2+} influx by nifedipine. Individual currents also possess different sensitivity to membrane potential and potassium channel blockers. I_O was found to be partially depressed by tetraethylammonium (TEA), quinidine and 4-aminopyridine (4-AP), I_T was blocked by low concentrations of TEA and quinidine but only by high 4-AP concentrations, while I_S was resistant to quinidine, 4-AP and was abolished by high TEA concentrations (Hisada *et al*., 1990). The STOCs and STICs recorded in the present study were similar to those obtained on smooth muscle cells of airways of other animal species and other smooth muscle tissues (Benham & Bolton, 1986; Ohya *et al*., 1987; Imaizumi *et al*., 1989; Muraki *et al*., 1990; Jansen & Sims, 1994). STOCs are believed to reflect the changes in submembraneous Ca^{2+} concentration, which may increase either as a result of Ca^{2+} influx or of a sudden transient release of Ca^{2+} from the stores adjacent to the plasma membrane (Benham & Bolton, 1986; Hisada *et al*., 1990; Bolton & Beech, 1992).

The effects of H_2O_2 on free $[Ca^{2+}]_i$, STOCs and background current were much less pronounced in Ca^{2+} free solution than in the presence of Ca^{2+} in the bathing fluid. A significant increase in free $[Ca^{2+}]_i$ occurred on readdition of Ca^{2+} to the bath in the continued presence of H_2O_2. This observation suggests two possible explanations: (i) H_2O_2 may cause a significant net Ca^{2+} influx, or (ii) a small Ca^{2+} influx may initiate the Ca^{2+} induced Ca^{2+} release. Roveri *et al*. (1992) showed that in vascular smooth muscle cells H_2O_2 caused a rapid increase in $[Ca^{2+}]_i$ followed by a decrease to a new constant level which was higher than the basal level before the oxidative challenge, suggesting that H_2O_2 may accelerate the Ca^{2+}-induced Ca^{2+} release.

The H_2O_2 induced increase in the magnitude of the currents elicited by ramp depolarizations support the conclusion that H_2O_2 may increase membrane conductance. Moreover, the non-linearity of the difference between currents elicited by ramp depolarization in the absence and presence of H_2O_2 indicated that the conductance underlying the H_2O_2-induced current was voltage-dependent. However, this does not exclude the participation of Ca^{2+}-induced Ca^{2+} release in the action of H_2O_2. Enhancement of the STOCs in the majority of the cells corresponded well with the time course of the increased free $[Ca^{2+}]_i$ and muscle contraction, suggesting that the primary event after application of H_2O_2 may be enhancement of intracellular Ca^{2+} concentration. The augmented potassium conductance was then a secondary event to the increase in free $[Ca^{2+}]_i$, which enhanced STOC activity, evoked a sustained outward current and a slight membrane hyperpolarization. Krippeit-Drews *et al*. (1995) came to a similar conclusion, they found that in the rat cultured artery H_2O_2 hyperpolarized the smooth muscle cell membrane. H_2O_2 which is highly permeable through membranes, most likely via oxidation of cytoplasmic inactivation gate cysteines (Vega-Saenz de Miera & Rudy, 1992) also affects several types of cloned voltage gated K^+ channels (e.g. expressed in *Xenopus* oocytes). Thus a direct effect of H_2O_2 on the K^+ channel could not be excluded.

In conclusion, the present study indicates that H_2O_2 increases intracellular free calcium ions, followed by an increase in the resting muscle tone, augments EFS or carbachol induced contractions and enhances Ca^{2+} and voltage-sensitive potassium conductance.

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References

BABIOR, B.M., KIPNES, R.S. & CURNUTTE, J.T. (1973). The production by leucocytes of superoxide, a potential bactericidal agent. *J. Clin. Invest*., **52,** 741–744.

BAST, A., HAENEN, G.R.M.M. & DOELMAN, C.J.A. (1991). Oxidants and antioxidants: State of the art. *Am. J. Med*., **91** (Suppl.3C), 3S–13S.

BLAKE, D.R., ALLEN, R.E. & LUNEC, J. (1987). Free radicals in biological systems: a review oriented to inflammatory processes. *Br. Med. Bull*., **43,** 371–385.

BENHAM, C.D. & BOLTON, T.B. (1986). Spontaneous transient outward currents in single visceral and vascular muscle cells of the rabbit. *J. Physol*., **381,** 385–406.

BOLTON, T.B. & BEECH, D.J. (1992). Smooth muscle potassium channels: their electrophysiology and function. In *Potassium Channel Modulators*. ed. Weston, A.H. & Hamilton, T.C. pp. 144–180. London: Blackwell Scientific Publications.

DE CHATELET, L.R., SHIRLEY, P.S., MCPHAIL, L.C., HUNTLEY, C.C., MUSS, H.B. & BASS, D.A. (1977). Oxidative metabolism of the human eosinophil. *Blood*, **50,** 526–535.

DRATH, D.B. & KARNOVSKY, M.L. (1975). Superoxide production by phagocytic leucocytes. *J. Exp. Med.*, **141,** 257–262.

GAO, Y. & VANHOUTTE, P.M. (1991). H_2O_2 induces epithelium-dependent contraction of canine bronchi. *Am. Rev. Respir. Dis.*, **143** (Suppl.), A341.

GAO, Y. & VANHOUTTE, P.M. (1993). Products of cyclooxygenase mediate the responses of the guinea pig trachea to hydrogen peroxide. *J. Appl. Physiol.*, **74,** 2105–2111.

GUPTA, J.B. & PRASAD, K. (1992). Mechanism of H_2O_2-induced modulation of airway smooth muscle. *Am. J. Physiol.*, **263,** L714–L722.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers. Arch.*, **391,** 85–100.

HARLAN, J.M. & CALLAHAN, K.S. (1984). Role of hydrogen peroxide in the neutrophil-mediated release of prostacyclin from cultured endothelial cells. *J. Clin. Invest.*, **74,** 442–448.

HISADA, T., KURACHI, Y. & SUGIMOTO, T. (1990). Properties of membrane currents in isolated smooth muscle cells from guinea-pig trachea. *Pflugers. Arch.*, **416,** 151–161.

HULSMANN, A.R., RAATGEEP, H.R., DEN HOLLANDER, J.C., STIJNEN, T., SAXENA, P.R., KERREBIJN, K.F. & DE JONGSTE, J.C. (1994). Oxidative epithelial damage produces hyperresponsiveness of human peripheral airways. *Am. J. Resp. Crit. Care. Med.*, **149,** 519–525.

IMAIZUMI, Y., MURAKI, K. & WATANABE, M. (1989). Ionic currents in single smooth muscle cells from the ureter of the guinea-pig. *J. Physiol.*, **441,** 131–159.

ITO, Y. (1991). Prejunctional control of excitatory neuroeffector transmission by prostaglandins in the airway smooth muscle tissue. *Am. Rev. Resp. Dis.*, **143,** S6–S10.

JANSSEN, L.J. & SIMS, S.M. (1994). Spontaneous transient inward currents and rhythmicity in canine and guinea-pig tracheal smooth muscle cells. *Pflügers Arch. Eur. J. Physiol.*, **427,** 473–480.

JING, L., INOUE, R., TASHIRO, K., TAKAHASHI, S. & ITO, Y. (1995). Role of nitric oxide in non-adrenergic, non-cholinergic relaxation and modulation of excitatory neuroeffector transmission in the cat airway. *J. Physiol.*, **483,** 225–237.

JOHNSON, K.J., FANTONE, J.C., KAPLAN, J. & WARD, P.A. (1981). In vivo damage of rat lungs by oxygen metabolites. *J. Clin. Invest.*, **67,** 983–993.

KATO, M., MORIKAWA, A., KIMURA, H., SHIMIZU, T., NAKANO, M. & KUROUME, T. (1991). Effects of antiasthma drugs on superoxide anion generation from human polymorphonuclear leucocytes or hypoxanthine-xanthine oxidase system. *Int. Arch. Allergy Appl. Immunol.*, **96,** 128–133.

KATSUMATA, U., MIURA, M., ICHINOSE, M., KIMURA, K., TAKAHASHI, T., INOUE, H. & TAKASHIMA, T. (1990). Oxygen radicals produce airway constriction and hyperresponsiveness in anesthetized cats. *Am. Rev. Respir. Dis.*, **141,** 1158–1161.

KRIPPEIT-DREWS, P., HABERLAND, FINGERLE, J., DREWS, G. & LANG, F. (1995). Effects of H_2O_2 on membrane potential and $[Ca^{2+}]_i$ of cultured rat arterial smooth muscle cells. *Biochem. Biophys. Res. Commun.*, **209,** 139–145.

MÁTYÁS, S. & BAUER, V. (1995). Involvement of epithelium and arachidonic acid metabolites in the mechanism of action of reactive oxygen species (ROS) on the guinea pig trachea. *Pharmacol. Res.*, **31** (Suppl.), 221.

MISAWA, M. & ARAI, H. (1993a). Airway inflammation induced by xanthine/xanthine oxidase in guinea pigs. *Agents Actions*, **38,** 19–26.

MISAWA, M. & ARAI, H. (1993b). Airway inflammatory effect of hydrogen peroxide in guinea pigs. *J. Toxicol. Envir. Health*, **38,** 453–448.

MORIKAWA, T., WEBBER, S.E. & WIDDICOMBE, J.G. (1991). The effect of hydrogen peroxide on smooth muscle tone, mucus secretion and epithelial albumin transport of the ferret trachea in vitro. *Pulm. Pharmacol.*, **2,** 106–113.

MURAKI, K., IMAIZUMI, Y., KOMIJA, T., KAWAI, T. & WATANABE, M. (1990). Effects of tetraethylammonium and 4-AP on outward currents and excitability in canine tracheal smooth muscle cells. *Br. J. Pharmacol.*, **100,** 507–515.

OHYA, Y., KITAMURA, K. & KURIYAMA, H. (1987). Cellular calcium regulates outward currents in rabbit intestinal smooth muscle cell. *Am. J. Physiol.*, **252,** C401–410.

OWEN, S., PEARSON, D., O DRISCOLL, R. & WOODCOCK, A. (1991). Evidence of free radical activity in asthma. *N. Engl. J. Med.*, **325,** 586–587.

RABE, K.F., DENT, G. & MAGNUSSEN, H. (1995). Hydrogen peroxide contracts human airways in vitro: role of epithelium. *Am. J. Physiol.*, **269,** L332–L338.

RAE, J., COOPER, K., GATES, G. & WATSKY, M. (1991). Low access resistance perforated patch recordings using amphotericin B. *J. Neurosci. Methods*, **37,** 15–26.

RHODEN, K.J. & BARNES, P.J. (1989). Effect of hydrogen peroxide on guinea-pig tracheal smooth muscle in vitro: role of cyclo-oxygenase and airway epithelium. *Br. J. Pharmacol.*, **98,** 325–330.

ROVERI, A., COASSIN, M., MAIORINO, M., ZAMBURLINI, A., VAN AMSTERDAM, F.T., RATTI, E. & URSINI, F. (1992). Effect of hydrogen peroxide on calcium homeostasis in smooth muscle cells. *Arch. Biochem. Biophys.*, **297,** 265–270.

SINCLAIR, A.J., BARNETT, A.H. & LUNEC, J. (1990). Free radicals and antioxidant systems in health and disease. *Br. J. Hosp. Med.*, **43,** 334–344.

SPORN, P.H., PETERS-GOLDEN, M. & SIMON, R.H. (1988). Hydrogen peroxide induced arachidonic acid metabolism in the rat alveolar macrophage. *Am. Rev. Resp. Dis.*, **137,** 49–56.

STEWART, R.M., WEIR, E.K., MONTGOMERY, M.R. & NIEWOEHNER, P.E. (1981). Hydrogen peroxide contracts airway smooth muscle: a possible endogenous mechanism. *Respir. Physiol.*, **45,** 333–342.

SZAREK, J.L. (1989). In vivo exposure to hyperoxia increases airway responsiveness in rats: demonstration in vivo and in vitro. *Am. Rev. Respir. Dis.*, **140,** 942–947.

SZAREK, J.L. & SCHMIDT, N.L. (1990). Hydrogen peroxide-induced potentiation of contractile responses in isolated rat airways. *Am. J. Physiol.*, **258,** L232–L237.

TAKAHASHI, T., MIURA, M., KATSUMATA, U., ICHINOSE, M., KIMURA, K., INOUE, H., TAKISHIMA, T. & SHIRATO, K. (1993). Involvement of superoxide in ozone-induced airway hyperresponsiveness in anesthetized cats. *Am. Rev. Respir. Dis.*, **148,** 103–106.

TAKAHASHI, N., TANAKA, H., ABDULLAH, N., JING, L., INOUE, R. & ITO, Y. (1995). Regional difference in the distribution of L-NAME sensitive and insensitive NANC relaxation in cat airway. *J. Physiol.*, **488,** 709–720.

VEGA-SAENZ DE MIERA, E. & RUDY, B. (1992). Modulation of K^+ channels by hydrogen peroxide. *Biochem. Biophys. Res. Commun.*, **186,** 1681–1687.

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