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Effects of H_2O_2 on membrane potential of smooth muscle cells in rabbit mesenteric resistance artery

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Abstract

The effects of H_2O_2 on the membrane potential of smooth muscle cells of rabbit mesenteric resistance arteries were investigated. H_2O_2 (3–30 μM) concentration-dependently hyperpolarized the membrane; this was inhibited by catalase but not by superoxide dismutase or the hydroxyl-radical scavenger dimethylthiourea. The cyclooxygenase inhibitor diclofenac partly inhibited the responses; the subsequent addition of the 5-lipoxygenase inhibitor 2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-*p*-benzoquinone (AA-861) (but not the cytochrome P_{450} inhibitor 17-octadecynoic acid) further attenuated H_2O_2-induced hyperpolarizations. The sarcolemmal ATP-sensitive K^+ (K_{ATP}) channel inhibitor 1-{5-[2-(5-chloro-*o*-anisamido)ethyl]-2-methoxyphenylsulfonyl}-3-methylthiourea, sodium salt (HMR-1098), blocked the H_2O_2-induced hyperpolarization in the absence and presence of diclofenac. H_2O_2 increased the production of prostaglandin E_2 and prostacyclin (estimated from its stable metabolite 6-keto-prostaglandin $F_{1\alpha}$), both of which produce a HMR-1098-sensitive hyperpolarization in the smooth muscle cells. It is concluded that, in smooth muscle cells of rabbit mesenteric artery, H_2O_2 increases the synthesis of vasodilator prostaglandins and possibly 5-lipoxygenase products, which produce a hyperpolarization by activating sarcolemmal K_{ATP} channels.

Keywords: Hydrogen peroxide; Hyperpolarization; ATP-sensitive K^+ channel; Prostaglandin; Smooth muscle vascular

1. Introduction

Reactive oxygen species, such as superoxide anions, hydrogen peroxide (H_2O_2) and hydroxyl radicals, are synthesized in variety of cells, endothelial cells, smooth muscle cells and fibroblasts, and possibly inflammatory cells, located in the walls of blood vessels (Griendling and Alexander, 1997). Of these, H_2O_2, which is known to be the most plentiful reactive oxygen metabolite that is able readily to penetrate cell membranes, is potentially harmful and thought to play a significant role in changes of vascular tone that occurs under certain types of pathophysiological conditions (Blake et al., 1987; Droge, 2001; Schubert and Wilmer, 1991). H_2O_2 produces a contraction in some vessels and a relaxation in others. For example, H_2O_2 contracts rat and rabbit aorta (Yang et al., 1998; Bharadwaj and Prasad, 1995), rat mesenteric artery (Gao and Lee, 2001) and rabbit pulmonary artery (Sheehan et al., 1993), while it relaxes canine middle cerebral, porcine coronary arteries (Iida and Katusic, 2000; Barlow et al., 2000) and cat cerebral arterioles (Wei et al., 1996). In preliminary experiments, we found that H_2O_2 (30 μM) relaxed endothelium-denuded strips of rabbit mesenteric resistance arteries that had been pre-contracted with noradrenaline (10 μM). The mechanism for the H_2O_2-induced relaxation has not yet been fully clarified, but it was recently suggested that, in porcine coronary artery, H_2O_2 produces a relaxation by hyperpolarizing the smooth muscle cells (Barlow et al., 2000). It is well known that the membrane hyperpolarization plays a significant role on the regulation of muscle contraction in resistance arteries (Kuriyama et al., 1998). Moreover, on the basis of the effects of a variety of K^+ channel blockers, it has been suggested that membrane hyperpolarization, induced by H_2O_2, is an important trigger for vasodilatation in the canine and cat cerebral arteries (Iida and Katusic, 2000; Wei et al., 1996). However, the mech-

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anisms underlying H_2O_2-induced hyperpolarizations have not been clarified in resistance arteries.

H_2O_2 activates phospholipase A_2, which in turn produces a variety of arachidonic acid metabolites in various types of cells (Chakraborti et al., 1989; Gao and Lee, 2001; Rao et al., 1995). It was recently suggested that, in smooth muscles of the porcine coronary artery, H_2O_2 produces a hyperpolarization through an action of synthesized lipoxygenase products (Barlow et al., 2000). In canine bronchi (Gao and Vanhoutte, 1992) and newborn piglet cerebral arterioles (Leffler et al., 1990), H_2O_2 increases the synthesis of both prostaglandin E_2 and prostacyclin; both hyperpolarize smooth muscle cells of resistance arteries (Parkington et al., 1993; Yamashita et al., 1999). Furthermore, cytochrome P_{450} metabolites of arachidonic acid also hyperpolarize some arterial smooth muscle cells (coronary, cerebral and renal arteries; Campbell and Harder, 1999). Thus, it remains to be clarified whether or not arachidonic acid metabolites play a role in H_2O_2-induced hyperpolarization and, if so, which arachidonic acid metabolites are responsible for this in the resistance arteries.

Using conventional microelectrode techniques, we have examined the effect of H_2O_2 on the membrane potential of smooth muscle cells of the rabbit mesenteric resistance artery, a vessel whose electrical and mechanical properties have been well characterized (Kuriyama et al., 1998; Yamashita et al., 1999). The role of arachidonic acid metabolites on the H_2O_2-induced hyperpolarization was pharmacologically characterized using selective inhibitors of this pathway. The K^+ channels responsible for the H_2O_2-induced hyperpolarization were characterized pharmacologically using a range of K^+ channel blockers. To test whether or not H_2O_2 does indeed increase arachidonic acid metabolism in these smooth muscle cells, the changes in the concentrations of prostaglandin E_2 and the stable prostacyclin metabolite 6-keto-prostaglandin $F_{1\alpha}$ were measured by enzyme-immunoassay in endothelium-denuded strips.

2. Materials and methods

2.1. Tissue preparation

Male Japan White albino rabbits (supplied by Kitayama Labes, Japan), weighing 1.9–2.4 kg, were anaesthetized by injection of pentobarbitone sodium (40 mg/kg, given intravenously) and then exsanguinated. The protocols used, which conformed with guidelines on the conduct of animal experiments issued by Nagoya City University Medical School, were approved by the Committee on the Ethics of Animal Experiments in Nagoya City University Medical School. The third and fourth branches of the mesenteric artery distributing to the region of the ileum (diameter: approximately 0.1–0.13 mm) were excised immediately and cleaned by removing connective tissue in Krebs solution under a binocular microscope at room temperature. After a given artery had been cut along its long axis, the endothelium was carefully removed by gentle rubbing of the internal surface of the strip using a small piece of razor blade, as described previously (Yamashita et al., 1999). Satisfactory ablation of the endothelium was verified pharmacologically by the absence of a hyperpolarization to acetylcholine (3 μM).

2.2. Recording of membrane potential changes

The membrane potential was measured using a conventional microelectrode technique, as reported previously (Yamashita et al., 1999). To allow recording of membrane potentials, each endothelium-denuded strip was pinned at both ends in a chamber, volume 0.7 ml, and superfused with warmed (36–37 °C) Krebs solution with a flow rate of about 2 ml/min. Glass microelectrodes were made from borosilicate glass tubing (OD = 1.2 mm with a glass filament inside; Hilgenberg, Malsfeld, Germany) and filled with 1 M KCl. The resistance of the electrodes was 80–180 MΩ. The electrode was inserted into smooth muscle cells from the adventitial side using a micromanipulator (model MHW-3; Narishige International, Tokyo, Japan). Membrane potentials, recorded using an Axoclamp-2B amplifier (Axon Instruments, Foster, CA, USA) were displayed on a cathode-ray oscilloscope (model VC-6020; Hitachi, Tokyo, Japan). Data were digitized at an acquisition rate of 200 Hz using an Axoscope 7.0/Digidata 1200 data-acquisition system (Axon Instruments) and stored on an IBM/AT compatible PC.

To determine the concentration-dependence of H_2O_2 (0.1–50 μM) on the membrane potential, a given concentration of H_2O_2 was applied for 3 min and followed by a washout period of 30 min (to allow the membrane potential to recover to the original level). This protocol was repeated using a range of concentrations of H_2O_2.

To examine the effects of superoxide dismutase, catalase and dimethylthiourea (a hydroxyl-radical scavenger) on the H_2O_2-induced membrane potential changes, H_2O_2 (30 μM) was first applied for 3 min (to record the control response), followed by a washout for 30 min. Superoxide dismutase (200 U/ml) for 10 min, catalase (800 U/ml) for 10 min or dimethylthiourea (1 mM) for 60 min was then applied, and H_2O_2 was re-applied in the presence of either agent. Each series was performed on a separate cell.

After the control H_2O_2 (30 μM) response had been recorded, the cyclooxygenase inhibitor diclofenac (3 μM) was applied for 60 min, then H_2O_2 was again applied in the presence of diclofenac. The effects of the 5-lipoxygenase inhibitor 2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-*p*-benzoquinone (AA-861) (10 μM) and the cytochrome P_{450} inhibitor 17-octadecynoic acid (3 μM) were examined as follows in diclofenac-treated strips. After pretreatment with diclofenac for 60 min, H_2O_2 (30 μM) was applied for 3 min, followed by a 30-min washout of H_2O_2. AA-861 for 30 min or 17-octadecynoic acid for 60

min was then applied before H_2O_2 was finally applied in the presence of diclofenac plus AA-861 or 17-octadecynoic acid. After the control H_2O_2 response had been recorded, the leukotriene C_4- and leukotriene D_4-receptor antagonist 4-oxo-8-[4-(4-phenylbutoxy)-benzoylamino]-2-tetrasol-5-yl)-4*H*-1-benzopyran hemihydrate (ONO-1078) (1 μM) was applied for 60 min, then H_2O_2 (30 μM) was again applied in the presence of ONO-1078. Each series was performed on a separate cell.

After recording the control H_2O_2 (30 μM) response (followed by a 30-min washout), iberiotoxin (0.1 μM) + apamin (0.1 μM), charybdotoxin (0.1 μM) + apamin (0.1 μM) or 4-aminopyridine (1 mM) was applied for 10 min. Similarly, glibenclamide [10 μM, a non-selective inhibitor of ATP-sensitive K^+ (K_{ATP}) channel] or 1-{5-[2-(5-chloro-*o*-anisamido)ethyl]-2-methoxyphenylsulfonyl}-3-methylthiourea, sodium salt (HMR-1098) (20 μM, a selective inhibitor of sarcolemmal K_{ATP} channel), was applied for 15 min, 5-hydroxydecanoic acid (1 mM, a selective inhibitor of mitochondrial K_{ATP} channel) was applied for 60 min or $BaCl_2$ (0.1 mM) for 5 min, then H_2O_2 was again applied in the presence of the K^+ channel inhibitor(s).

The effects of HMR-1098 on the H_2O_2-induced hyperpolarization in diclofenac-treated strips was examined as follows. After pretreatment with diclofenac (3 μM) for 60 min, H_2O_2 (30 μM) was applied for 3 min, followed by a 30-min washout of H_2O_2. HMR-1098 (20 μM) was then applied for 15 min before H_2O_2 was finally applied in the presence of diclofenac plus HMR-1098.

After pretreatment with diclofenac (3 μM) for 60 min, prostaglandin E_2 (0.1 μM), beraprost (a stable analogue of prostacyclin, 0.1 μM) (Yamashita et al., 1999), leukotriene C_4 (1 μM) or leukotriene D_4 (1 μM) was applied for 2 min. When the effect of HMR-1098 on the response to prostaglandin E_2 (0.1 μM) or beraprost (0.1 μM) was to be examined, the control response to the agent was first recorded (followed by a 30-min washout) in diclofenac-treated strips. HMR-1098 (20 μM) was then applied for 15 min before prostaglandin E_2 or beraprost were applied in the presence of diclofenac and HMR-1098. Each series was performed on a separate cell.

2.3. Assay for prostaglandin E_2, prostacyclin and cyclic GMP

After equilibration for 2 h, endothelium-denuded strips were transferred into a tube containing 0.4 ml Krebs solution with guanethidine (0.5 μM) and equilibrated for 1 h at 36 °C. H_2O_2 (30 μM) was then added for 5 min. For the assays of prostaglandin E_2 and 6-keto-prostaglandin $F_{1\alpha}$, solution, taken from the tube, was assayed using an enzyme-immunoassay kit (Amersham Pharmacia Biotech, Tokyo, Japan), as described previously (Suzuki et al., 1991; Yamashita et al., 1999). To assay for cyclic GMP, the reaction was halted by soaking the strips in ice-cold 8% trichloroacetic acid. Strips were then homogenized in a solution containing trichloroacetic acid in a glass homogenizer, the homogenate centrifuged and the pellet used for the protein assay, which was by a modified Lowry assay procedure (DC Protein Assay Kit; Bio-Rad, Hercules, CA, USA) using bovine serum albumin as standard. The supernatant fraction was treated with water-saturated ether three times and assayed for cyclic GMP using an enzyme-immunoassay kit (Amersham Pharmacia Biotech).

2.4. Solutions

The ionic composition of the Krebs solution was as follows (mM): Na^+ 137.4, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.6, HCO_3^- 15.5, $H_2PO_4^-$ 1.2, Cl^- 134 and glucose 11.5. The solutions were bubbled with 95% O_2 and 5% CO_2, and their pH was maintained at 7.4. High K^+ (40 mM) solution was made by isotonic replacement of NaCl with KCl. All the

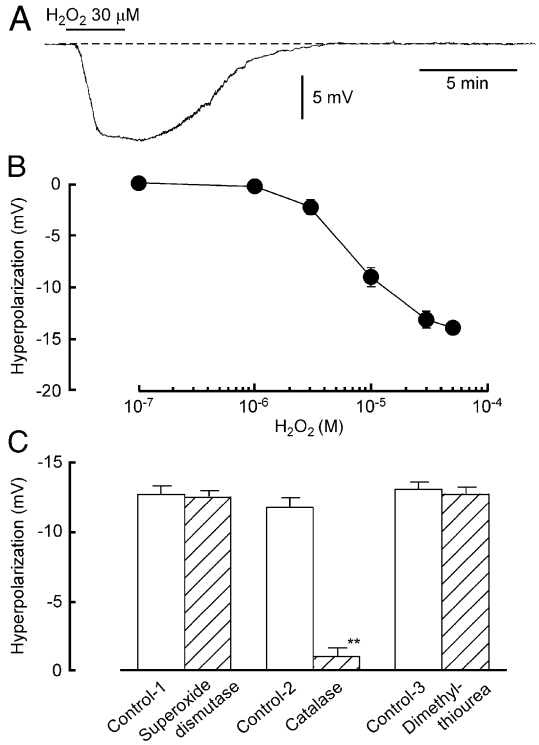

Fig. 1. Effect of H_2O_2 on membrane potential of smooth muscle cells of rabbit mesenteric artery. (A) Representative tracing of the effect of H_2O_2 (30 μM) on membrane potential. H_2O_2 was applied for 3 min as indicated by the bar. Broken line indicates resting membrane potential level. (B) Concentration-dependent effects of H_2O_2 (0.1 – 50 μM). Mean of data from 8 to 20 strips, with S.E.M. shown by vertical line (where it exceeds the diameter of the symbol). (C) Summary of the effects of superoxide dismutase ($n = 4$), catalase ($n = 4$) and dimethylthiourea ($n = 5$) on the hyperpolarization induced by 30 μM H_2O_2. The H_2O_2-induced response was first observed before application of superoxide dismutase (200 U/ml), catalase (800 U/ml) or dimethylthiourea (1 mM), and the second H_2O_2-induced response was then observed in the presence of either agent in the same cell. 'Control 1' represents the control H_2O_2 response obtained before application of superoxide dismutase. 'Control 2' is the control response for catalase and 'Control 3' for dimethylthiourea. Data are shown as mean ± S.E.M. $^{**}P < 0.01$ vs. corresponding control (Student's paired *t*-test).

solutions used in the present experiments contained guanethidine (5 μM, to prevent sympathetic-transmitter release).

2.5. Drugs

Drugs used were acetylcholine hydrochloride (Daiichi Pharmaceutical, Tokyo, Japan), catalase (from bovine liver), superoxide dismutase (Cu^{2+}/Zn^{2+} type, from bovine erythrocytes), AA-861, barium chloride ($BaCl_2$) and 4-aminopyridine (Wako, Osaka, Japan); 17-octadecynoic acid, glibenclamide, diclofenac sodium and 5-hydroxydecanoic acid (Sigma, St. Louis, MO, USA); dimethylthiourea (Nacalai, Kyoto, Japan); iberiotoxin, charybdotoxin and apamin (Peptide Institute, Osaka, Japan); and guanethidine (Tokyo Kasei, Tokyo, Japan). Beraprost sodium was kindly provided by Yamanouchi Pharmaceutical (Tokyo, Japan); prostaglandin E_2, leukotriene C_4, leukotriene D_4 and ONO-1078 by Ono Pharmaceutical (Osaka, Japan); and HMR-1098 by Aventis Pharmaceuticals (Frankfurt, Germany). Glibenclamide, HMR-1098, AA-861 and ONO-1078 were dissolved in dimethyl sulphoxide (Dojin, Kumamoto, Japan). The final concentration of dimethyl sulphoxide when diluted in Krebs solution was 0.06% at maximum. This concentration of dimethyl sulphoxide affected neither the resting membrane potential nor the H_2O_2-induced hyperpolarization. Prostaglandin E_2, leukotriene C_4 and leukotriene D_4 were each dissolved in absolute ethanol (as a stock solution, stored at -80 °C) and further dilution was made in Krebs solution just before use. Dimethylthiourea was directly dissolved in Krebs solution. The other drugs were dissolved in ultra-pure Milli-Q water (Japan Millipore, Tokyo, Japan) to make stock solutions.

2.6. Statistics

All values are expressed as mean ± S.E.M.; n denotes the number of strips (which equals the number of animals). Statistical significance was determined using Student's paired and unpaired t-tests. Difference were considered to be significant at $P < 0.05$.

3. Results

3.1. Effect of H_2O_2 on membrane potential

The resting membrane potential of the smooth muscle cells was -60.7 ± 0.8 mV ($n = 80$). H_2O_2 (3–30 μM) hy-

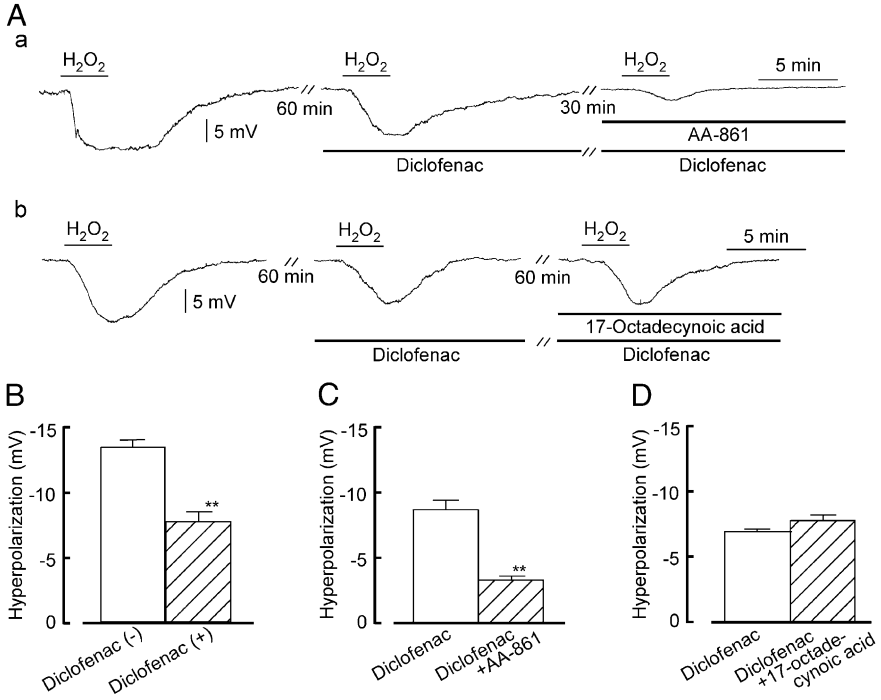

Fig. 2. Effects of inhibitors of arachidonic acid metabolism on H_2O_2 (30 μM)-induced hyperpolarization. (A) Representative tracings of the effect of cyclooxygenase inhibitor diclofenac (3 μM) and the effects of combined application of diclofenac and 5-lipoxygenase inhibitor AA-861 (10 μM) (Aa) or cytochrome P_{450} inhibitor 17-octadecynoic acid (3 μM) (Ab) on the H_2O_2-induced hyperpolarization. The H_2O_2 (30 μM)-induced response was first observed before application of each inhibitor (left panels of (Aa) and (Ab)), the second H_2O_2-induced response was observed after 60-min application of diclofenac (middle panels of (Aa) and (Ab)) and the final H_2O_2-induced response was observed after 30-min application of AA-861 (right panel of (Aa)) or 60-min application of 17-octadecynoic acid (right panel of (Ab)) in the presence of diclofenac. In a given panel, recordings were made from same cell. Traces in (Aa) and (Ab) were obtained from different strips. (B) Summary of the effect of diclofenac (3 μM) on the H_2O_2-induced hyperpolarization ($n = 12$). (C) The effect of AA-861 (10 μM) in the presence of diclofenac ($n = 8$). (D) The effect of 17-octadecynoic acid (3 μM) in the presence of diclofenac ($n = 4$). Data are shown as mean ± S.E.M. $^{**}P < 0.01$ vs. corresponding control response (Student's paired t-test).

Table 1
Effects of H_2O_2, prostaglandin E_2, beraprost, leukotriene C_4 and leukotriene D_4 on membrane potential in smooth muscle cells in the absence and presence of HMR-1098

	Membrane potential changes (mV)	
	Before application of HMR-1098	After application of HMR-1098
H_2O_2 (30 μM)	− 7.9 ± 0.5 (4)	− 1.7 ± 0.8 (4)[a]
Prostaglandin E_2 (0.1 μM)	− 13.8 ± 0.4 (4)	− 3.9 ± 1.2 (4)[a]
Beraprost (0.1 μM)	− 18.4 ± 0.3 (4)	− 2.8 ± 0.4 (4)[a]
Leukotriene C_4 (1 μM)	− 0.2 ± 0.1 (4)	–
Leukotriene D_4 (1 μM)	− 0.4 ± 0.4 (5)	–

All data were obtained after 60-min pretreatment of diclofenac (3 μM). Data are expressed as mean ± S.E.M. The number of strips is given in parenthesis. (–) Not measured.
[a] $P<0.01$ vs. before application of HMR-1098 (20 μM).

perpolarized the membrane in a concentration-dependent manner (Fig. 1A and B). Catalase (800 U/ml) did not change the resting membrane potential ($n=4$, $P>0.5$), but it blocked the H_2O_2 (30 μM)-induced hyperpolarization ($P<0.001$). Neither superoxide dismutase (200 U/ml) nor the hydroxyl-radical scavenger dimethylthiourea (1 mM) (Wasil et al., 1987) modified the resting membrane potential ($n=4–5$, $P>0.3$), nor did they alter the H_2O_2 (30 μM)-induced hyperpolarization ($n=4–5$, $P>0.5$) (Fig. 1C).

3.2. Effects of diclofenac and AA-861 on H_2O_2-induced hyperpolarization

Diclofenac (3 μM, a cyclooxygenase inhibitor) did not change the resting membrane potential ($n=12$, $P>0.3$), but it attenuated the hyperpolarization induced by 30 μM H_2O_2 ($P<0.0001$) (Fig. 2A and B). Diclofenac, AA-861 (10 μM, a 5-lipoxygenase inhibitor) (Ashida et al., 1983), also failed to alter the resting membrane potential ($n=8$, $P>0.9$), but it further attenuated the H_2O_2-induced hyperpolarization ($P<0.0001$; Fig. 2Aa and C). By contrast, diclofenac, 17-octadecynoic acid (3 μM, a cytochrome P_{450} inhibitor) (Dong et al., 1997), altered neither the resting membrane potential ($n=4$, $P>0.2$) nor the H_2O_2-induced hyperpolarization ($P>0.1$; Fig. 2Ab and D).

3.3. Effects of prostaglandin E_2, beraprost, leukotriene C_4, leukotriene D_4 and sodium nitroprusside on membrane potential

Prostaglandin E_2 (0.1 μM) and beraprost (0.1 μM) each produced a hyperpolarization (Table 1). By contrast, neither leukotriene C_4 (1 μM) nor leukotriene D_4 (1 μM) significantly modified the membrane potential. ONO-1078 (1 μM, a leukotriene C_4- and leukotriene D_4-receptor antagonist) (Fujiwara et al., 1993) did not modify the H_2O_2-induced hyperpolarization, the H_2O_2 (30 μM)-induced hyperpolarizations being 11.1 ± 1.0 and 10.9 ± 1.3 mV in the absence and presence of ONO-1078, respectively ($n=4$, $P>0.5$).

Sodium nitroprusside (50 μM) did not alter the membrane potential (resting potential − 60.1 ± 0.5 and − 61.0 ± 1.2 mV before and after application of 50 μM sodium nitroprusside, respectively; $n=5$, $P>0.1$).

3.4. Effects of K^+ channel blockers on H_2O_2-induced hyperpolarization

High K^+ (40 mM) depolarized the membrane (by 31.6 ± 1.9 mV, $n=4$) and blocked the hyperpolarization induced by 30 μM H_2O_2. Glibenclamide (10 μM), a non-selective inhibitor of K_{ATP} channel, depolarized the membrane ($P<0.01$) and significantly attenuated the H_2O_2-induced hyperpolarization ($P<0.0001$; Table 2). K_{ATP} channels are present on the sarcolemmal and inner mitochondrial membranes (Noma, 1983; Inoue et al., 1991) and H_2O_2 has been suggested to act on those located in the mitochondrial membrane in cardiomyocytes (Zhang et al., 2002). To study whether or not mitochondrial K_{ATP} channels play a role in the H_2O_2-induced hyperpolarization in smooth muscle cells

Table 2
Effects of K^+ channel inhibitors on resting membrane potential (RMP) and H_2O_2 (30 μM)-induced hyperpolarization

	In the absence of blocker (mV)		In the presence of blocker (mV)	
	RMP	H_2O_2 hyperpolarization	RMP	H_2O_2 hyperpolarization
Glibenclamide (10 μM)	− 62.0 ± 1.7 (8)	11.0 ± 1.0 (8)	− 57.5 ± 1.3 (8)[a]	3.2 ± 0.7 (8)[b]
HMR-1098 (20 μM)	− 59.2 ± 1.0 (10)	10.1 ± 1.1 (10)	− 55.1 ± 0.6 (10)[a]	3.4 ± 0.6 (10)[b]
5-Hydroxydecanoic acid (1 mM)	− 57.3 ± 0.6 (4)	15.1 ± 1.0 (4)	− 55.0 ± 0.4 (4)	14.8 ± 0.2 (4)
4-Aminopyridine (1 mM)	− 63.4 ± 0.7 (5)	10.4 ± 1.3 (5)	− 53.5 ± 0.7 (5)[a]	14.1 ± 1.0 (5)[c]
Charybdotoxin (0.1 μM) + apamin (0.1 μM)	− 58.4 ± 1.5 (5)	10.6 ± 0.9 (5)	− 56.2 ± 2.1 (5)[d]	12.6 ± 0.3 (5)
Iberiotoxin (0.1 μM) + apamin (0.1 μM)	− 59.3 ± 0.3 (4)	13.3 ± 0.5 (4)	− 51.4 ± 0.4 (4)[a]	13.6 ± 0.5 (4)
Ba^{2+} (0.1 mM)	− 56.3 ± 0.5 (7)	13.5 ± 0.8 (7)	− 51.0 ± 0.4 (7)[a]	13.6 ± 1.1 (7)

Data are expressed as mean ± S.E.M. The number of strips is given in parenthesis.
[a] $P<0.01$ vs. before application of K^+ channel blockers.
[b] $P<0.01$ vs. before application of K^+ channel blockers.
[c] $P<0.05$ vs. before application of K^+ channel blockers.
[d] $P<0.05$ vs. before application of K^+ channel blockers.

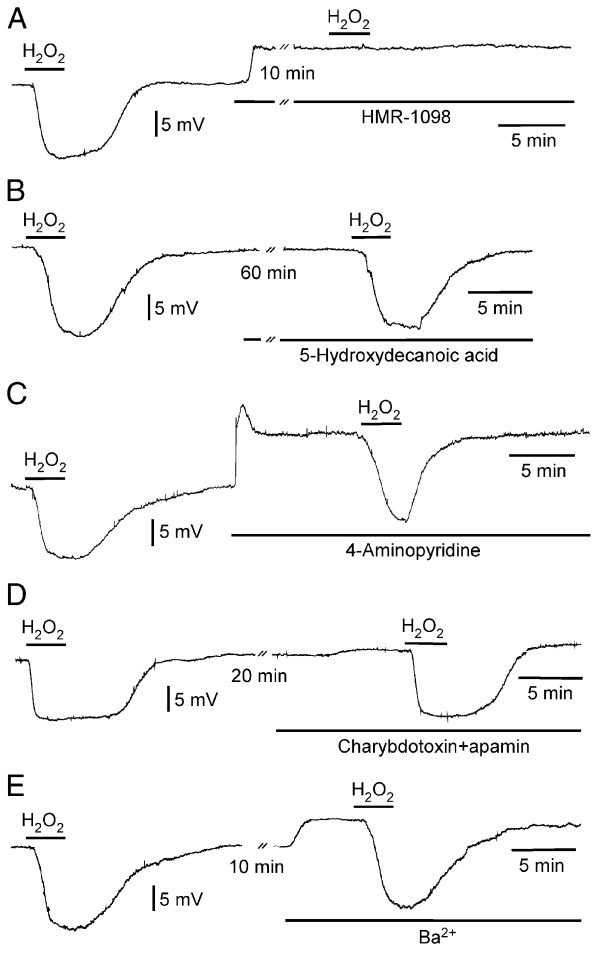

Fig. 3. Representative tracings of the effects of various K^+ channel inhibitors on H_2O_2 (30 μM)-induced hyperpolarization. (A) The effect of the sarcolemmal K_{ATP} channel inhibitor HMR-1098 (20 μM). (B) The effect of the mitochondrial K_{ATP} channel inhibitor 5-hydroxydecanoic acid (1 mM). (C) The effect of the K_V channel inhibitor 4-aminopyridine (1 mM). (D) The effect of the K_{Ca} channel inhibitor charybdotoxin (0.1 μM) + apamin (0.1 μM). (E) The effect of the K_{ir} channel inhibitor Ba^{2+} (0.1 mM). In a given panel, recordings were made from one and the same cell. Traces in (A) to (E) were obtained from different strips. H_2O_2 (30 μM) was applied for 3 min and each inhibitor was pretreated as indicated by the bars.

of the rabbit mesenteric artery, we investigated the effects of HMR-1098 (a selective inhibitor of sarcolemmal K_{ATP} channel; Sato et al., 2000) and 5-hydroxydecanoic acid (a selective inhibitor of mitochondrial K_{ATP} channel; Sato et al., 2000) on the H_2O_2 (30 μM)-induced hyperpolarization. HMR-1098 (20 μM) caused membrane depolarization ($P<0.01$) and inhibited the H_2O_2-induced hyperpolarization to the same extent as glibenclamide (10 μM) ($P<0.001$; Table 2 and Fig. 3A). The inhibitory action of HMR-1098 was also observed on the H_2O_2-induced remaining hyperpolarization in the presence of diclofenac ($P<0.01$; Table 1). In addition, HMR-1098 also significantly inhibited the hyperpolarizations induced by prostaglandin E_2 (0.1 μM, $P<0.01$) and beraprost (0.1 μM, $P<0.001$) (Table 1). In contrast, 5-hydroxydecanoic acid (1 mM) modified neither the resting membrane potential nor the H_2O_2-induced hyperpolarization ($P>0.1$; Table 2 and Fig. 3B).

4-Aminopyridine [1 mM, a voltage-dependent K^+ (K_V) channel blocker] (Nelson and Quayle, 1995) depolarized the membrane ($P<0.01$) and enhanced the H_2O_2-induced hyperpolarization ($P<0.05$; Table 2 and Fig. 3C). Apamin (0.1 μM, an inhibitor of small-conductance K_{Ca} channels) + charybdotoxin (0.1 μM, an inhibitor of intermediate-conductance K_{Ca} channels) (Garcia et al., 1997) depolarized the membrane ($P<0.05$), but had no effect on the H_2O_2-induced hyperpolarization ($P>0.05$; Table 2 and Fig. 3D). Similarly, apamin (0.1 μM) + iberiotoxin (0.1 μM, an inhibitor of large-conductance K_{Ca} channels) (Garcia et al., 1997) depolarized the membrane ($P<0.001$), but did not modify the H_2O_2-induced hyperpolarization ($P>0.05$; Table 2). Likewise, Ba^{2+} [0.1 mM, an inhibitor of inwardly rectifying K^+ (K_{ir}) channels] depolarized the membrane ($P<0.0001$), but did not modify the H_2O_2-induced hyperpolarization ($P>0.1$; Table 2 and Fig. 3E).

3.5. Effects of H_2O_2 on synthesis of prostaglandin E_2 and 6-keto-prostaglandin $F_{1\alpha}$

Under resting conditions, the concentrations of prostaglandin E_2 and 6-keto-prostaglandin $F_{1\alpha}$ were 5.1 ± 0.6 ($n=9$) and 47.7 ± 13.9 pg/mg protein ($n=9$), respectively. H_2O_2 (30 μM) significantly increased each concentration: prostaglandin E_2 to 40.6 ± 8.2 pg/mg protein ($n=9$, $P<0.001$) and 6-keto-prostaglandin $F_{1\alpha}$ to 365.6 ± 44.0 pg/mg protein ($n=9$, $P<0.001$) (Fig. 4).

3.6. Effects of H_2O_2 on synthesis of cyclic GMP

The basal concentration of cyclic GMP was 44.3 ± 11.7 fmol/mg protein ($n=8$) and H_2O_2 (30 μM) did not significantly modify this (52.3 ± 27.8 fmol/mg protein, $n=8$, $P>0.5$).

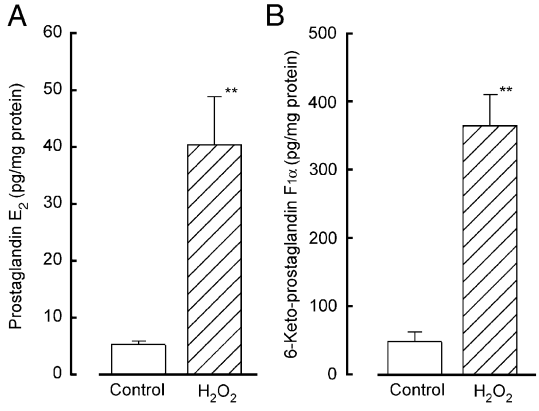

Fig. 4. Effect of H_2O_2 (30 μM) on production of prostaglandin E_2 and 6-keto-prostaglandin $F_{1\alpha}$ in endothelium-denuded strips. (A) The effect of H_2O_2 on the production of prostaglandin E_2 ($n=9$). (B) The effect of H_2O_2 on the production of 6-keto-prostaglandin $F_{1\alpha}$ ($n=9$). Control indicates values obtained in the absence of H_2O_2. Data are shown as mean ± S.E.M. $^{**}P<0.01$ vs. corresponding control (Student's paired t-test).

4. Discussion

H_2O_2 is produced by the dismutation of superoxide anion (by superoxide dismutase) and converted into a hydroxyl radical in the presence of reduced transition metals (e.g., ferrous or cuprous irons) (Chance et al., 1979). In the present study, H_2O_2 produced a hyperpolarization that was inhibited by catalase, but not by either superoxide dismutase or the hydroxyl-radical scavenger dimethylthiourea. These results indicate that, in the rabbit mesenteric resistance artery, H_2O_2 hyperpolarizes the smooth muscle cells directly.

4.1. Role of arachidonic acid metabolites in H_2O_2-induced hyperpolarization

H_2O_2 increases phospholipase A_2 activity, which in turn increases the release of arachidonic acid in cultured aortic smooth muscle cells (Rao et al., 1995). Various arachidonic acid metabolites (such as prostaglandin E_2, prostacyclin, epoxyeicosatrienoic acids and lipoxygenase metabolites) can produce a hyperpolarization in arterial smooth muscle cells (Kuriyama et al., 1998). It was recently found that H_2O_2 produces a hyperpolarization through an action of unidentified lipoxygenase products in smooth muscle cells of the porcine coronary artery (Barlow et al., 2000). In the present experiments, we found that, in smooth muscle cells of the rabbit mesenteric artery, (a) the cyclooxygenase inhibitor diclofenac attenuated the H_2O_2-induced hyperpolarization (by about one half) and (b) the 5-lipoxygenase inhibitor AA-861, but not cytochrome P450-monooxygenase inhibitor 17-octadecynoic acid, reduced the response remaining in the presence of diclofenac (by two thirds). Furthermore, in accord with the previous findings in newborn piglet cerebral arterioles (Leffler et al., 1990) and canine bronchi (Gao and Vanhoutte, 1992), H_2O_2 increased the production of both prostaglandin E_2 and prostacyclin (estimated from the production of 6-keto-prostaglandin $F_{1\alpha}$) in endothelium-denuded strips. Moreover, prostaglandin E_2 (0.1 μM) and the stable prostacyclin analogue beraprost, but not leukotriene C_4 or leukotriene D_4, each hyperpolarized the smooth muscle cell membrane. The suggestion that leukotriene C_4 and leukotriene D_4 play no significant role in the H_2O_2-induced hyperpolarization is supported by our finding that ONO-1078 (a leukotriene C_4- and leukotriene D_4-receptor antagonist) had no effect on H_2O_2-induced responses. Although the synthesis of thromboxane A_2 was found to be increased by H_2O_2 in rat mesenteric artery (Gao and Lee, 2001), a contribution of thromboxane A_2 to H_2O_2-induced hyperpolarization in smooth muscle cells of rabbit mesenteric arteries is unlikely since a thromboxane A_2 mimetic, 9,11-dideoxy-11α,9α-epoxy-methano-prostaglandin $F_{2\alpha}$ (U-46619), produces a depolarization (rather than a hyperpolarization) in smooth muscle cells of guinea-pig coronary artery (Parkington et al., 1995). Taken together, these results suggest that H_2O_2 produces a hyperpolarization via the action of synthesized vasodilator prostaglandins and possibly of 5-lipoxygenase products (other than leukotriene C_4 and leukotriene D_4) in smooth muscle cells of the rabbit mesenteric resistance artery.

4.2. Pharmacological characterization of K^+ channels responsible for the H_2O_2-induced hyperpolarization

H_2O_2 has been shown to modulate the opening a number of K^+ channels in a variety of cells. In human umbilical endothelial cells, patch-clamp experiments have shown that H_2O_2 modulates two different types of K^+ channels depending on its concentration: at low concentrations (10–250 μM), it inhibits inward-rectifying K^+ channels (K_{ir}) but at higher concentrations (>500 μM) it activates Ca^{2+}-activated K^+ (K_{Ca}) channels (Bychkov et al., 1999). Activation of K_{Ca} channels by high concentrations of H_2O_2 (0.1–1 mM) was demonstrated in smooth muscle cells of porcine coronary arteries (Barlow et al., 2000; Hayabuchi et al., 1998). In the present experiments, H_2O_2 (30 μM) produced a hyperpolarization that was inhibited by glibenclamide (a non-selective inhibitor of K_{ATP} channels) but not by apamin (an inhibitor of small-conductance K_{Ca} channels) plus either charybdotoxin (an inhibitor of intermediate-conductance K_{Ca} channels) or iberiotoxin (an inhibitor of large-conductance K_{Ca} channels). Furthermore, an inhibitor of neither K_{ir} channels (Ba^{2+}) nor K_v channels (4-aminopyridine) inhibited the hyperpolarization. Thus, in accord with previous findings in renal epithelial LLC-PK_1 cells (Filipovic and Reeves, 1997) and in mouse pancreatic β-cells (Krippeit-Drews et al., 1999), our results suggest that, in smooth muscle cells of the rabbit mesenteric resistance artery, H_2O_2 (3–30 μM) produces the hyperpolarization through an activation of K_{ATP} channels. The hypothesis is also consistent with the previous pharmacological evidence found in cat cerebral arterioles (Wei et al., 1996).

It has been suggested that H_2O_2 may open the K_{ATP} channels located in the mitochondrial membrane in ventricular myocytes from chick embryos (Zhang et al., 2002). In the present experiments, the H_2O_2-induced hyperpolarization was inhibited by HMR-1098 (a selective inhibitor of sarcolemmal K_{ATP} channels) to an extent similar to that induced by glibenclamide (a non-selective inhibitor of K_{ATP} channels), but this was not modified by 5-hydroxydecanoic acid (a selective inhibitor of mitochondrial K_{ATP} channels). These results suggest that the sarcolemmal K_{ATP} channel is the target responsible for the H_2O_2-induced hyperpolarization in smooth muscle cells of the rabbit mesenteric resistance artery.

In bovine pulmonary arteries, H_2O_2 was found to increase the cellular concentration of cyclic GMP (Burke and Wolin, 1987), which activates both K_{ATP} and K_{Ca} channels so producing a hyperpolarization in smooth muscle cells of the rat arteriole (Zhao et al., 2000). However, H_2O_2 (30 μM) did not significantly modify the

concentration of cyclic GMP in endothelium-denuded strips of the rabbit mesenteric arteries. Furthermore, a nitric oxide (NO) donor sodium nitroprusside (50 μM) did not modify the membrane potential in the smooth muscle cells. Moreover, no important role of cyclic GMP on H_2O_2-induced vasodilation was found in canine middle cerebral arteries (Iida and Katusic, 2000) and cat cerebral arterioles (Wei et al., 1996). Taken together, these observations suggest that in smooth muscle cells of rabbit mesenteric arteries, cyclic GMP does not play a significant role in the H_2O_2-induced hyperpolarization.

In conclusion, in the smooth muscle cells of the rabbit mesenteric resistance artery, H_2O_2 hyperpolarizes the cell membrane through a direct action. It is suggested that H_2O_2 increases the synthesis of vasodilator prostaglandins (prostaglandin E_2 and prostacyclin) and possibly 5-lipoxygenase products and that all of these activate sarcolemmal K_{ATP} channels, thus producing a hyperpolarization of the smooth muscle cells.

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