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Receptor tyrosine and MAP kinase are involved in effects of H_2O_2 on interstitial cells of Cajal in murine intestine

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Abstract

Hydrogen peroxide (H_2O_2) is involved in intestinal motility through changes of smooth muscle activity. However, there is no report as to the modulatory effects of H_2O_2 on interstitial cells of Cajal (ICC). We investigated the H_2O_2 effects and signal transductions to determine whether the intestinal motility can be modulated through ICC. We performed whole-cell patch clamp in cultured ICC from murine intestine and molecular analyses. H_2O_2 hyperpolarized the membrane and inhibited pacemaker currents. These effects were inhibited by glibenclamide, an inhibitor of ATP-sensitive K^+ (K_{ATP}) channels. The free-radical scavenger catalase inhibited the H_2O_2-induced effects. MAFP and $AACOCF_3$ (a cytosolic phospholipase A_2 inhibitors) or SC-560 and NS-398 (a selective COX-1 and 2 inhibitor) or AH6809 (an EP_2 receptor antagonist) inhibited the H_2O_2-induced effects. PD98059 (a mitogen activated/ERK-activating protein kinase inhibitor) inhibited the H_2O_2-induced effects, though SB-203580 (a p38 MAPK inhibitor) or a JNK inhibitor did not affect. H_2O_2-induced effects could not be inhibited by LY-294002 (an inhibitor of PI_3-kinases), calphostin C (a protein kinase C inhibitor) or SQ-22536 (an adenylate cyclase inhibitor). Adenoviral infection analysis revealed H_2O_2 stimulated tyrosine kinase activity and AG 1478 (an antagonist of epidermal growth factor receptor tyrosine kinase) inhibited the H_2O_2-induced effects. These results suggest H_2O_2 can modulate ICC pacemaker activity and this occur by the activation of K_{ATP} channels through PGE_2 production *via* receptor tyrosine kinase-dependent MAP kinase activation.

Keywords: hydrogen peroxide • interstitial cells of Cajal • pacemaker currents • cyclooxygenase • receptor tyrosine kinase • MAP kinase

Introduction

Intestinal inflammation either in human beings or in experimental animal models is associated with altered gastrointestinal motility [1–3]. The mechanisms of altered motility have been associated with morphological and functional changes in smooth muscle and enteric nerves [4–6]. Large numbers of polymorphonuclear leucocytes that generate reactive oxygen species (ROS) such as H_2O_2 are found in the mucosa and submucosa of the inflamed bowel disease, suggesting that H_2O_2 plays an integral role in the inflammatory process [7]. Several *in vitro* studies have reported that exposure to H_2O_2 alters gastrointestinal smooth muscle contractility. For example, H_2O_2 decreases sigmoid smooth muscle contractility in ulcerative colitis patients and the radical scavenger catalase prevents ulcerative colitis-induced reduction of muscle contractions [8, 9]. Exposure to H_2O_2 reduces the lower esophageal sphincter tone in human esophagitis and treatment with catalase can restore the lower esophageal tone to normal [10]. From above findings, it is suggested that H_2O_2 may be an important mediator causing dysmotility in intestinal inflammation.

The gastrointestinal smooth muscles show spontaneous mechanical contractions. These contractions are mediated by the generation of periodic membrane depolarization (slow waves). It is well known that interstitial cells of Cajal (ICC) are pacemaker cells that generate slow waves, which are initiated by spontaneous inward currents (pacemaker currents) [11–13], even if some reports suggested that gastrointestinal pacing was possible without

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ICC and other pacemaker cells may be involved in pacing activity [14, 15]. ICC are coupled to each other and to smooth muscle cells *via* gap junctions. ICC also express various receptors for receiving inhibitory and excitatory signals from the enteric nervous systems [16, 17]. Acetic acid-induced inflammation reduces the membrane potential and reduces the amplitude and duration of slow waves in colonic circular muscle cells, suggesting that ICC may involve in motility changes in the inflammatory process [18].

Despite the observation that H_2O_2 is involved in intestinal motility through the changes of smooth muscle contractility, ion channel activity and enteric neuronal mechanisms, there are no reports describing the modulatory effects of H_2O_2 on pacemaker activities of ICC. In the present study, we investigated the effects of H_2O_2 on pacemaker currents and signal transductions to determine whether the intestinal motility can be modulated by ROS through ICC in the murine intestine.

Materials and methods

Preparation of cells and tissues

Balb/C mice (8- to 13-day old) of either sex were anaesthetized with ether and killed by cervical dislocation. The small intestines from 1 cm below the pyloric ring to the cecum were removed and opened along the mesenteric border. The luminal contents were washed away with Krebs-Ringer bicarbonate solution. The tissues were pinned to the base of a Sylgard dish and the mucosa was removed by sharp dissection. Small stripes of intestinal muscle were equilibrated in Ca^{2+}-free Hank's solution for 30 min and the cells were dispersed with an enzyme solution containing collagenase (Worthington Biochemical Co, Lakewood, NJ, USA), 1.3 mg/ml, bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA), 2 mg/ml, trypsin inhibitor (Sigma), 2 mg/ml and ATP, 0.27 mg/ml. Cells were plated onto sterile glass coverslips coated with murine collagen (2.5 μg/ml, Falcon/BD) in 35-mm culture dishes. The cells were then cultured at 37°C in a 95% O_2–5% CO_2 incubator in SMGM (smooth muscle growth medium, Clonetics Corp., San Diego, CA, USA) supplemented with 2% antibiotics/antimycotics (Gibco, Grand Island, NY, USA) and 5 ng/ml murine stem cell factor (SCF, Sigma).

Patch-clamp experiments

Cultures of cells contained single cells and networks of cells that had gross morphological properties similar to ICC *in situ,* including fusiform cell bodies, large, prominent nuclei with little perinuclear cytoplasm and multiple, thin processes extending from the nuclear region that were often interconnected with processes of neighbouring cells [19]. Recordings were made from ICC with the patch-clamp technique as soon as the network-like structures. Recordings were made from cells within networks that had morphologies similar to the cells that were immunopositive for c-Kit.

The whole-cell configuration of the patch-clamp technique was used to record membrane currents (voltage clamp) and membrane potentials (current clamp) from cultured ICC. Currents or potentials were amplified by use of an Axopatch 1-D (Axon Instruments, Foster City, CA, USA). Command pulse was applied using an IBM-compatible personal computer and pClamp software (version 6.1; Axon Instruments). The data were filtered at 5 kHz and displayed on a computer monitor, and a pen recorder (Gould 2200, Gould, Valley view, OH, USA). All experiments were performed at 30°C.

Results were analysed using pClamp and Graph Pad Prizem (version 2.01) software.

Protein tyrosine kinase assays using adenoviral vectors

Adenoviral vectors encoding enhanced green fluorescent fusion proteins were generated by the two-plasmid rescue system. The following scheme shows a typical protocol for the adenoviral vector assay in live cells using adenoviral vector transduction.

> Day 1: Seed appropriate ICC in culture medium with the adenoviral vector at a predetermined multiplicity of infection (MOI) and incubate for 24 hrs
> Day 2: H_2O_2 compounds are added to the incubated samples and the samples are allowed to incubate at 37°C. After incubation, the nuclei of ICC are stained using 2.5 μM Hoechst stain.

Image acquisition (EGFP redistribution assays) was performed on a confocal microscope (×100; fluoviews 300, Olympus) using 360/40 nm (HoechstTM) and 475/20 nm (EGFP) excitation filters and a 535/20 nm emission filter.

Solutions and drugs

The cells were bathed in a solution containing 5 mm KCl, 135 mm NaCl, 2 mm $CaCl_2$, 10 mm glucose, 1.2 mm $MgCl_2$ and 10 mm HEPES, adjusted to pH 7.2 with Tris. The pipette solution contained 140 mm KCl, 5 mm $MgCl_2$, 2.7 mm K_2ATP, 0.1 mm Na_2GTP, 2.5 mm creatine phosphate disodium, 5 mm HEPES, 0.1 mm EGTA, adjusted to pH 7.2 with Tris.

The drugs used were: hydrogen peroxide, catalase, SC560, $AACOCF_3$, AH6809, glibenclamide, PD98059, SB203580, SQ-22536, JNK inhibitor, NS398, MAFP, LY-294002. PD98059 and JNK inhibitor were purchased from Calbiochem Co., and the other compounds were purchased from the Sigma Chemical Co. (San Diego, CA, USA).

Statistical analysis

Data are expressed as the means ± standard errors. Differences in the data were evaluated by the Student's t-test. A *P* values less than 0.05 were taken as a statistically significant difference.

The *n* values reported in the text refer to the number of cells used in the patch-clamp experiments.

Results

Effects of H_2O_2 on the pacemaker activity in ICC

To determine whether H_2O_2 have function on pacemaker activities of ICC, we recorded the pacemaker potential in a current clamp

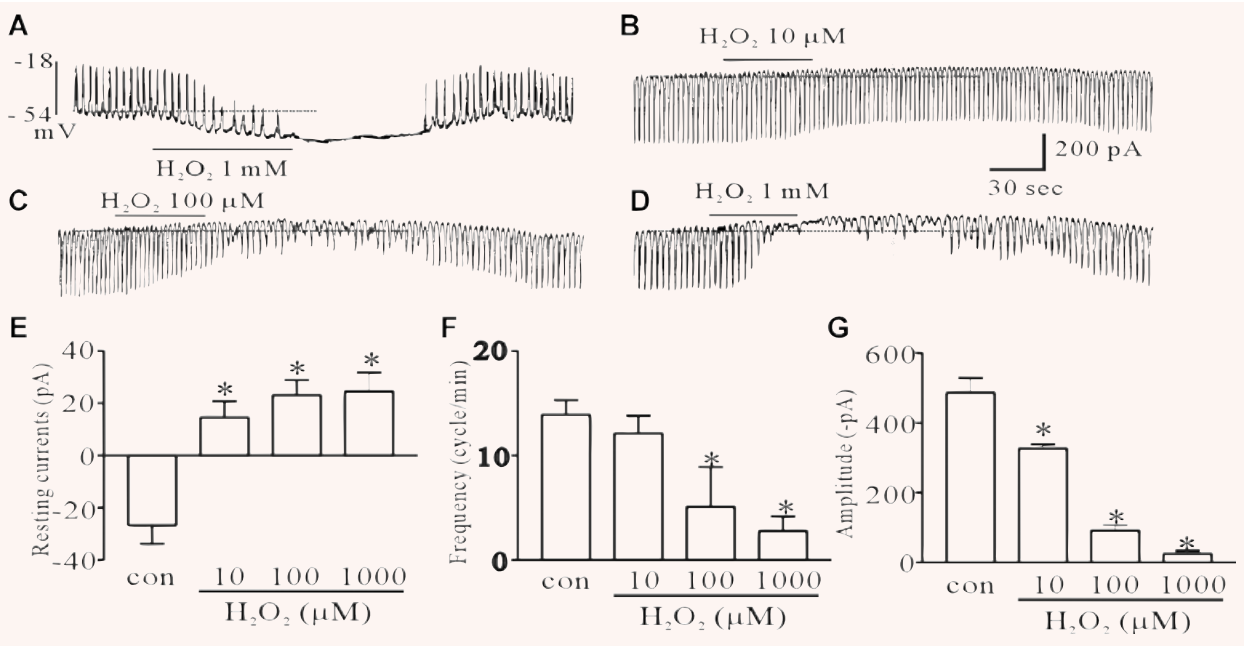

Fig. 1 The effect of H_2O_2 on pacemaker potentials and currents in cultured ICC of the murine intestine. (**A**) Pacemaker potentials of ICC exposed to 1-mM H_2O_2 in the current clamping mode (I = 0). (**B**, **C** and **D**) Pacemaker currents of ICC exposed to H_2O_2 (10, 100 μM and 1 mM) at a holding potential of −70 mV. (**E**, **F** and **G**) A summarized bar graph showing the H_2O_2-induced effects on pacemaker currents of ICC. The bars represent mean ± S.E. values (n = 7/group). Asterisks indicate significantly different from the controls ($P < 0.05$), and the dotted lines indicate zero current levels.

and pacemaker currents in a voltage clamp. Under the current clamp mode, H_2O_2 (1 mM) produced membrane hyperpolarization and decreased the amplitude of the pacemaker potential (Fig. 1A). Under control conditions at $I = 0$, the resting membrane potential was −52 ± 4.8 mV, and the amplitude of the pacemaker potential was 39.6 ± 5 mV. In the presence of H_2O_2, the membrane was hyperpolarized to −76.3 ± 8.0 mV and the amplitude of the pacemaker potentials decreased to 7.6 ± 3.9 mV (n = 6, data not shown). Under a voltage clamp at a holding potential of −70 mV, the ICC generated spontaneous inward currents. The mean frequency of these pacemaker currents was 13.9 ± 1.4 cycles/min and their mean amplitude and mean resting current levels were −416 ± 42 pA and −26 ± 7 pA, respectively (n = 7). The addition of 10 μM H_2O_2 slightly reduced the amplitude and frequency of pacemaker currents and slightly increased resting currents in the outward direction (Fig. 1B). In the presence of 100 μM and 1 mM H_2O_2, the pacemaker currents were largely inhibited and the resting currents were increased in the outward direction (Fig. 1C and D). The inhibitory frequencies and amplitudes with H_2O_2 treatment were 5.1 ± 1.8 cycles/min and −90.9 ± 16 pA at a concentration of 100 μM H_2O_2 and 2.8 ± 1.4 cycles/min and −24 ± 19 pA at a concentration of 1 mM H_2O_2, respectively. The resting current levels were 23 ± 5.9 pA at a concentration of 100 μM H_2O_2 and 24.5 ± 7.2 pA at a concentration of 1 mM H_2O_2 (n = 7) (Fig. 1E–G). Next, to determine whether H_2O_2 affects K_{ATP} channels in ICC, we used the K_{ATP} channels inhibitor glibenclamide, after or before H_2O_2 treatment. The H_2O_2-induced effects on pacemaker currents were inhibited by co- or pretreatment with glibenclamide (Fig. 2A and E), indicating that H_2O_2 may activate K_{ATP} channels in ICC. The results of glibenclamide treatment on the H_2O_2-induced effects on pacemaker currents are summarized in Figure 2B–D.

Effects of catalase and phospolipase A_2 inhibitors on the H_2O_2-induced inhibition of pacemaker currents

To evaluate whether the H_2O_2-induced inhibition of pacemaker currents was mediated *via* a ROS and PLA_2 signal pathway, we treated the ICC with catalase (3000 unit/ml), MAFP (10 μM) and $AACOCF_3$ (10 μM) before exposure to H_2O_2. Catalase inhibited the H_2O_2-induced effects (Fig. 3A) indicating that the H_2O_2-induced effects may be an ROS-dependent mechanism. Figure 3B and C shows that the 1 mM H_2O_2-induced effects were significantly inhibited by treatment with MAFP and $AACOCF_3$. As shown in Figure 3C–E, the values of the frequency, amplitude and resting

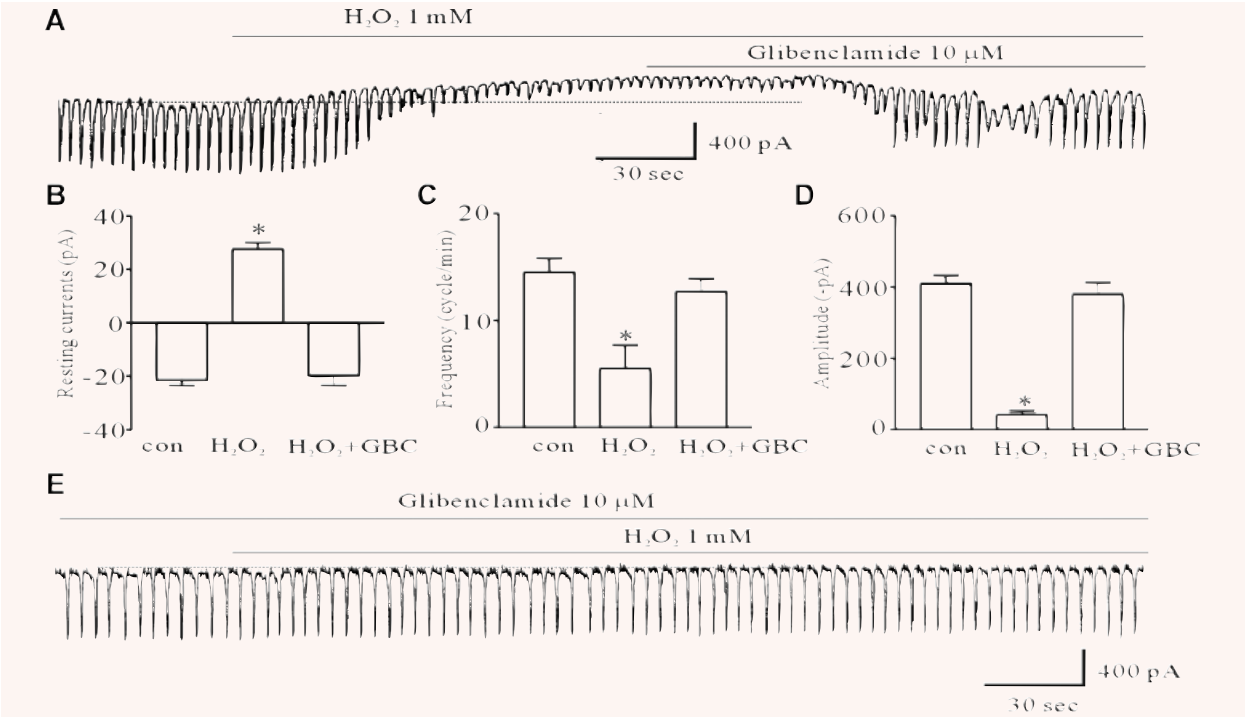

Fig. 2 The effect of glibenclamide, an inhibitor of ATP-sensitive K^+ channels, on H_2O_2-induced action on pacemaker currents of ICC from the murine intestine. (**A**) Pacemaker currents exposed to 1 mM H_2O_2 at a holding potential of −70 mV. The H_2O_2-induced effects were reversed by adding 10 μM glibenclamide. (**B**, **C**, and **D**) The inhibitory response to glibenclamide on the H_2O_2-induced action on pacemaker currents. (**E**) The effect of 1-mM H_2O_2 on pacemaker currents after pretreating cells with 10 μM glibenclamide. The bars represent mean ± S.E. values ($n = 6$). Asterisks indicate significantly different from the controls ($P < 0.05$), and the dotted lines indicate zero current levels. glibenclamide, GBC.

currents by H_2O_2 in the presence of catalase, MAFP and $AACOCF_3$ were significantly different from those obtained in the absence of catalase, MAFP and $AACOCF_3$ ($n = 5$).

Involvement of cyclooxygenase and the prostaglandin E_2 receptor in the H_2O_2-induced inhibition of pacemaker currents

Since activation of the PLA_2 pathway would stimulate the cyclooxygenase (COX) and prostaglandins synthesis, we examined the role of this system in mediating the H_2O_2-induced effects using SC-560 (10 μM), a specific COX-1 inhibitor, or NS-398 (10 μM), a specific COX-2 inhibitor. ICC were pretreated with SC-560 (10 μM) or NS-398 (10 μM) prior to the treatment with H_2O_2. We found that the H_2O_2-induced effects was inhibited by pretreatment with SC-560 or NS-398 ($n = 7$, Fig. 4A and B). Our previous report suggested that PGE_2-activated K_{ATP} channels through PGE-EP_2 receptor activation in ICC [20]. Therefore, we tested AH6809 (10 μM), a PGE_2-EP_2 receptor antagonist and found the H_2O_2-induced effects were inhibited by pretreatment with AH6809 (Fig. 5A). Furthermore, AH6809 inhibited the PGE_2-induced effects (Fig. 5B). The values of the frequency, amplitude and resting currents induced by H_2O_2 in the presence of AH6809 were significantly different from those obtained in the absence of AH-6809 ($n = 6$, Fig. 5C–E). These results suggest that PGE_2 may be involved in the H_2O_2-induced inhibition of pacemaker currents.

Involvement of mitogen-activated protein kinases (MAPKs) in the H_2O_2-induced inhibition of pacemaker currents

Since many reports suggested H_2O_2 activate MAPKs in many cell types, we investigated whether MAPKs are involved in the H_2O_2-induced effects using PD98059, a p44/42 MAPK inhibitor, or SB203580, a p38 MAPK inhibitor, or a JNK (c-jun NH_2-terminal kinse) inhibitor. Figure 6A shows that PD98059 (10 μM) prevented the H_2O_2-induced effects. However, SB203580 and JNK inhibitor did not affect the H_2O_2-induced effects (Fig. 6B and C). The values of the frequency, amplitude and resting currents by H_2O_2 in the presence of PD98059 were significantly different from those obtained in the absence of PD98059 ($n = 6$, Fig. 6E–G). In

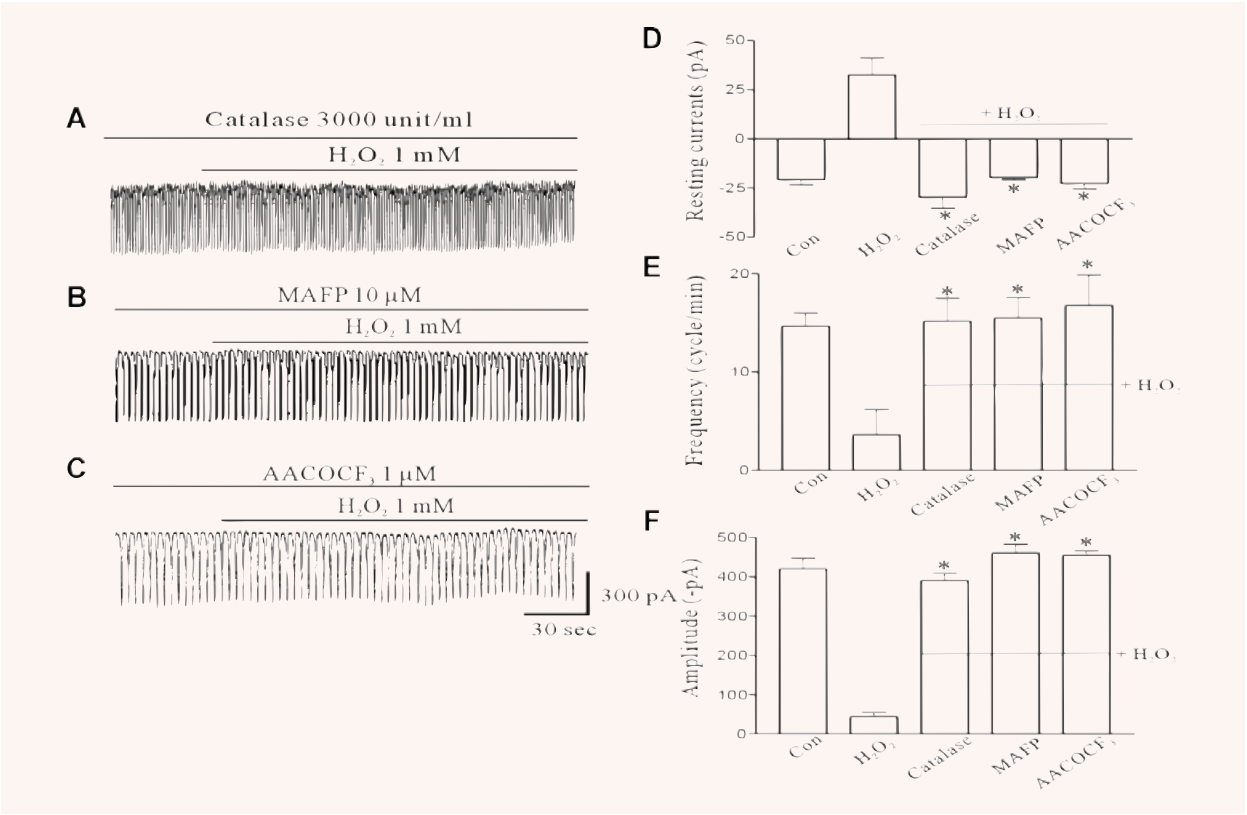

Fig. 3 The effect of catalase, a radical scavenger, MAFP, a non-specific phospholipase A_2 inhibitor, and $AACOCF_3$, a cytosolic phospholipase A_2 inhibitor on the H_2O_2-induced action on pacemaker currents of ICC from the murine intestine. (**A**) The effect of 1 mM H_2O_2 on pacemaker currents after pretreating cells with catalase (3000 units/ml). (**B**) Pacemaker currents exposed to 1 mM H_2O_2 after pretreating cells with 10 μM MAFP at a holding potential of −70 mV. (**C**) The effect of 1 mM H_2O_2 on pacemaker currents after pretreating cells with 10 μM $AACOCF_3$. (**D**, **E** and **F**) The inhibitory response to catalase, MAFP or $AACOCF_3$ on the H_2O_2-induced effects on pacemaker currents. The bars represent mean ± S.E. values ($n = 6$). Asterisks indicate significantly different from H_2O_2 alone ($P < 0.05$).

addition, PD98059 inhibited the PGE_2-induced effects; supporting the role of PGE_2 as a mediator of H_2O_2 (Fig. 6D).

Effects of the adenylate cyclase inhibitor, PI3-kinase inhibitor and protein kinase C inhibitor in the H_2O_2-induced inhibition of pacemaker currents

To determine an upstream regulator of p44/42 activation, ICC were pretreated with either 10 μM SQ-22536 (an adenylate cyclase inhibitor), 10 μM LY-294002 (a PI3-kinase inhibitor) and 0.1 μM calphostin C (a PKC inhibitor). We found that all inhibitors did not affect the H_2O_2-induced effects ($n = 5$: bar graph not shown), indicating that the H_2O_2-induced effects in ICC are part of a cAMP-, PI3-kinase- and PKC-independent pathway (Fig. 7A–C).

Involvement of EGFR (epidermal growth factor receptor) tyrosine kinase in the H_2O_2-induced inhibition of pacemaker currents

To evaluate the role of tyrosine kinase in the H_2O_2-induced effects, ICC were infected with adenovirus that was constructed containing a tyrosine kinase gene. In Figure 7D, we could see the tyrosine kinase domain was located predominantly in the nucleus of ICC. When ICC were treated with H_2O_2 (1 mM) for 30 min, there was a translocation of the tyrosine kinase from nucleus to the cytosol (Fig. 7E). This finding indicates that H_2O_2 stimulates the tyrosine kinase activity in ICC. Furthermore, to determine whether the H_2O_2-induced inhibition of pacemaker currents is mediated by EGFR tyrosine kinase, we treated the cells with AG 1478, a potent and selective inhibitor of EGFR tyrosine kinase. AG 1478 (10 μM) inhibited the H_2O_2-induced effects (Fig. 7F). Also, we found that AG 1478 inhibited the PGE_2-induced inhibition of pacemaker currents

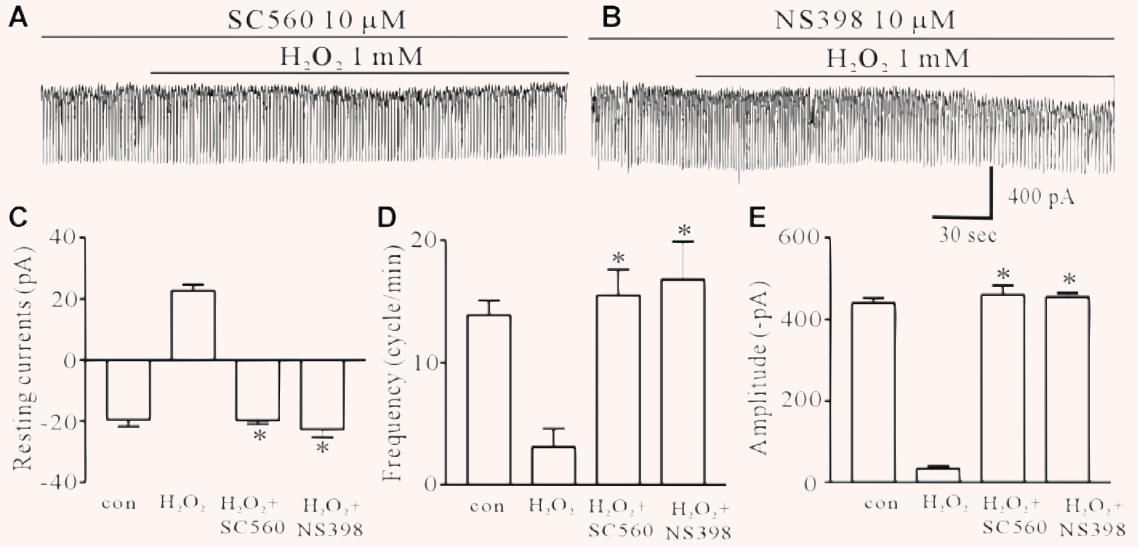

Fig. 4 The effect of SC-560, a specific cyclooxgenase-1 inhibitor, and NS-398, a specific cyclooxygenase-2 inhibitor, on the H_2O_2-induced effects on pacemaker currents of ICC from the murine intestine. (**A**) Pacemaker currents exposed to 1 mM H_2O_2 after pretreating cells with 10 μM SC-560 at a holding potential of −70 mV. (**B**) The effect of 1 mM H_2O_2 on pacemaker currents after pretreating cells with 10 μM NS-398. (**C**, **D** and **E**) The inhibitory response to SC-560 or NS-398 on the H_2O_2-induced action on pacemaker currents. The bars represent mean ± S.E. values ($n = 7$). Asterisks indicate significantly different from H_2O_2 alone ($P < 0.05$).

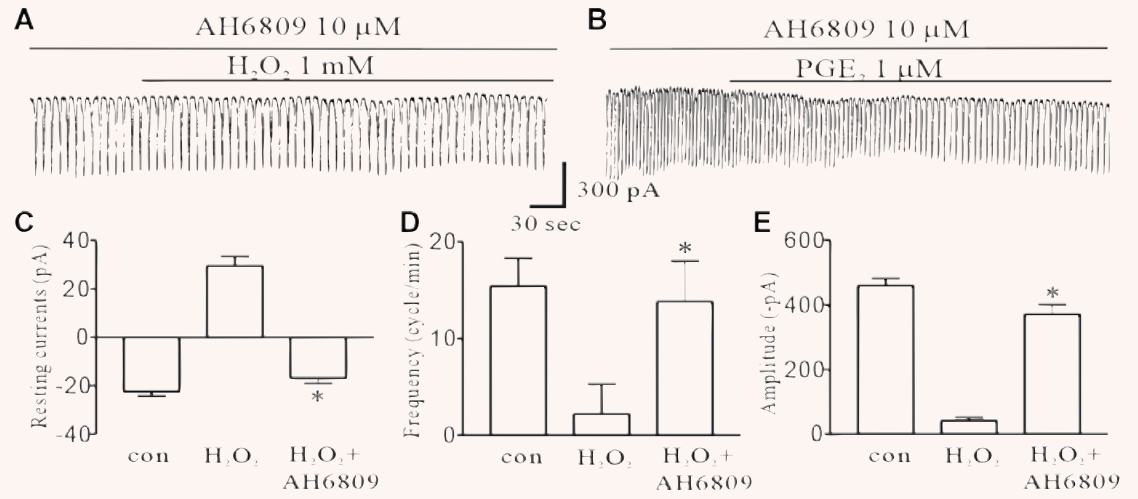

Fig. 5 The effect of AH6809, a PGE_2-EP_2 receptor antagonist, on the H_2O_2-induced effects on pacemaker currents of ICC from the murine intestine. (**A**) The effect of 1 mM H_2O_2 on pacemaker currents after pretreating cells with 10 μM AH6809. (**B**) The effect of 1 μM PGE_2 on pacemaker currents after pretreating cells with 10 μM AH6809. (**C**, **D** and **E**) The inhibitory response to AH6809 on the H_2O_2-induced effects on pacemaker currents. The bars represent mean ± S.E. values ($n = 6$). Asterisks indicate significantly different from H_2O_2 alone ($P < 0.05$).

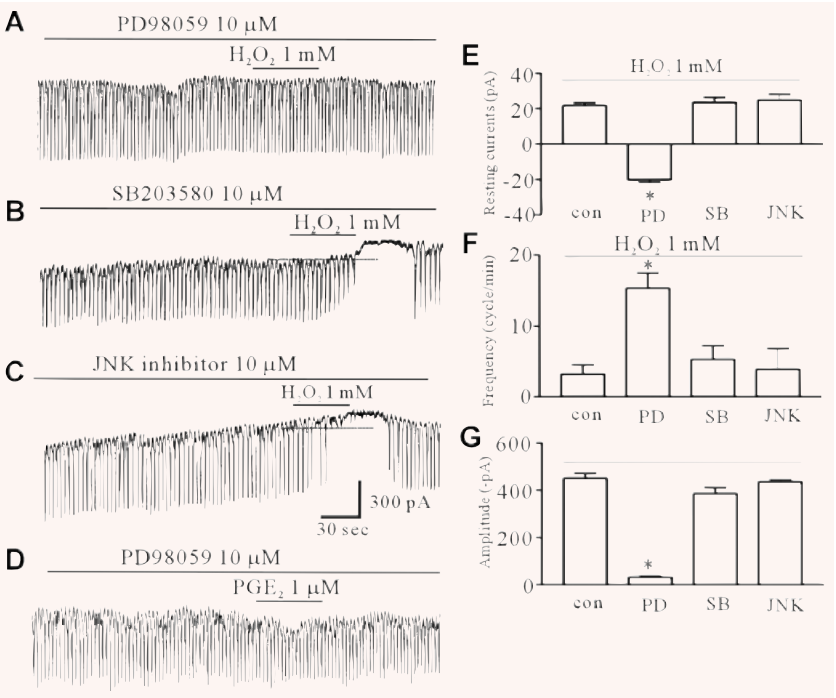

Fig. 6 The effect of PD98059, SB203580 and the JNK inhibitor on the H_2O_2-induced effects on pacemaker currents of ICC from the murine intestine. (**A**) The effect of 1 mM H_2O_2 on pacemaker currents after pretreating cells with 10 μM PD98059, a p44/42 MAPK inhibitor, for 15 min. (**B**) The effect of 1 mM H_2O_2 on pacemaker currents after pretreating cells with 10 μM SB203580, a p38 MAPK inhibitor, for 15 min. (**C**) The effect of 1 mM H_2O_2 on pacemaker currents after pretreating cells with 10 μM JNK inhibitor, c-jun NH_2-terminal kinase inhibitor, for 15 min. (**D**) The effect of 1 μM PGE_2 on pacemaker currents after pretreating cells with 10 μM PD98059 for 15 min. (**E**, **F**, and **G**) The inhibitory response to PD98059 on the H_2O_2-induced action on pacemaker currents. The bars represent mean ± S.E. values (n = 6/group). Asterisks indicate significantly different from H_2O_2 alone ($P < 0.05$), and the dotted lines indicate zero current levels. PD98059, PD; SB203580, SB; JNK inhibitor, JNK.

(Fig. 7G). This finding suggests that the H_2O_2-induced effect is dependent on EGFR tyrosine kinase activation through PGE_2.

Discussion

In the present study, we first demonstrated that H_2O_2 induces hyperpolarization of the membrane and inhibits pacemaker currents in intestinal ICC. These effects are mediated through activation of K_{ATP} channels by COX-dependent PGE_2 production, and receptor tyrosine and MAP kinase are involved in the H_2O_2-induced process.

K^+ channels play an important role in regulating cellular excitability in various cell types. H_2O_2 hyperpolarizes the resting membrane potential through the activation of several K^+ channels in vascular and visceral smooth muscles [21, 22]. The activation of K_{ATP} channels would lead to membrane hyperpolarization, which is thought to be an important mechanism for smooth muscle relaxation. We have already functionally reported that ICC have an K_{ATP} channels and that the ICC are modulated by bile salts and antidepressants [23, 24]. Deoxycholic acid inhibited pacemaker currents that were blocked by glibenclamide, an inhibitor of the K_{ATP} channel, and imipramine blocked the pinacidil (an opener of the K_{ATP} channels)-induced inhibition in a similar manner as glibenclamide. K_{ATP} channels is comprised of Kir 6.2 with SUR2B in cultured ICC of the mouse [20], indicating these channels may play an important role in the electrical activities of ICC and be a target of endogenous substances and drugs, as in smooth muscle. In the present study, H_2O_2 hyperpolarized the membrane potential and inhibited pacemaker currents. These effects were inhibited by glibenclamide, which indicates that H_2O_2 can change pacemaker activities through the activation of K_{ATP} channels in ICC.

H_2O_2 mediates the production of PGs from arachidonic acid *via* COX enzyme activation in GI tract. For examples, exposure to H_2O_2 causes damage to the plasma membrane of the gallbladder muscle and contraction through the generation of PGE_2 by the $cPLA_2$-cyclooxygenase pathway [25]. H_2O_2 reduces the lower esophageal sphincter (LES) tone in human esophagitis and the cat LES tone by increasing the synthesis of COX-2 and PGE_2 [10, 26]. And we reported that deoxycholic acid activated COX-2-dependent PGE_2 production [23]. The biological effects of PGE_2 are mediated *via* four different receptor subtypes (EP1, -2, -3 and -4) [27]. We found that PGE_2 action on pacemaker currents in ICC was mediated by EP2 receptors and the expression of the EP2 subtype was only detected [20]. In the present study, H_2O_2-induced effects were blocked by $cPLA_2$ inhibitors (MAFP and $AACOCF_3$) or COX inhibitors (SC560 and NS-398) as well as by an EP2-receptor antagonist (AH6809). Taken together, these results suggest that the inhibition of pacemaker currents through activation of K_{ATP} channels by H_2O_2 is mediated by $cPLA_2$-COX-dependent PGE_2 production.

A remaining issue to consider is the determination of the signal linkage between production of PGE_2 by H_2O_2 and the activation of K_{ATP} channels. The MAPKs signaling pathway plays an important role in the mediation of cellular responses including visceral smooth muscle contraction [28]. Three principal

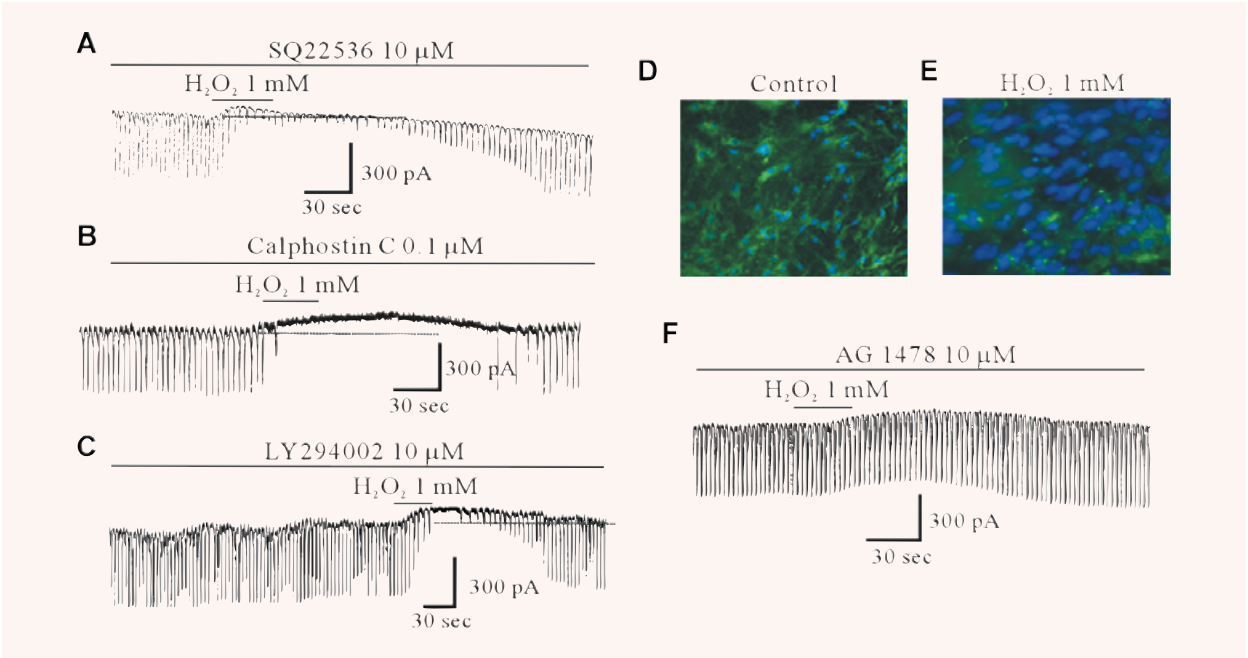

Fig. 7 The effect of SQ-22536, calphostin C, LY-294002 and AG1478 on the H_2O_2-induced effects on pacemaker currents of ICC and images from the ICC expressing the tyrosine kinase fusion protein, delivered by an adenoviral vector from the murine intestine. (**A**) The effect of 1 mM H_2O_2 on pacemaker currents after pretreating cells with 10 μM SQ22536, an adenylate cyclase inhibitor, for 10 min. (**B**) The effect of 1 mM H_2O_2 on pacemaker currents after pretreating cells with 0.1 μM calphostin C, protein kinase C inhibitor, for 10 min. (**C**) The effect of 1 mM H_2O_2 on pacemaker currents after pretreating cells with 10 μM LY-294002, PI3-kinase inhibitor, for 15 min. (**D**) In resting cells: a punctuate pattern of fluorescence is observed. (**E**) When ICC are treated with 1 mM H_2O_2 for 30 min., the ICC becomes diffuse. (**F**) The effect of 1 mM H_2O_2 on pacemaker currents after pretreating cells with 10 μM AG1478, a potent and selective inhibitor of epidermal growth factor receptor, for 15 min. (**G**) The effect of 1 μM PGE_2 on pacemaker currents after pretreating cells with 10 μM AG1478. The dotted lines indicate zero current levels.

MAPKs are expressed in various tissues: p44/42 MAPK, JNK and p38 MAPK [29]. H_2O_2 and PGE_2 activate MAPKs in many cell types [30–32]. In the present study, PD98059, an inhibitor of p44/42 MAPK, inhibited the H_2O_2- or PGE_2-induced inhibition of pacemaker currents suggesting p44/42 MAPK may be involved in the modulation of pacemaker currents by H_2O_2. It has been reported that H_2O_2 induced ERK1/2 activation in cultured feline ileal smooth muscle cells and pulmonary arterial smooth muscle cells [32, 33]. In addition, MAPK-ERK is involved in the activation of K_{ATP} channel by nitric oxide in neurons [34]. In addition, PGE_2 activates ERK1/2 in several cells [35, 36], which supports our results.

cAMP, PI3-kinase, PKC and tyrosine kinase are upstream regulators in MAPKs activation [22, 37]. The cAMP pathway is the major inhibitory mechanism responsible for the relaxation of gastrointestinal smooth muscle [38]. In addition, K_{ATP} channels are opened by the cAMP-dependent signal pathway in smooth muscle cells [39]. However, in the current study, treatment with an adenylate cyclase inhibitor had no influence on the H_2O_2-induced effects. Also, cell permeable 8-bromo-cAMP did not inhibit pacemaker currents [40], indicating cAMP is not involved in the H_2O_2-induced effects. An antagonist of PI3-kinase, LY-294002 suppresses H_2O_2-induced contraction in vascular smooth muscle cells [41]. PI3-kinase also has been reported to mediate ERK1/2 activation by insulin and thrombin [42, 43]. PI3 kinase leads to activation of PKC [44]. In addition, H_2O_2 has been reported to stimulate PKC activities [45]. Also, some reports suggested that spontaneous rhythmic contractions of uterus and intestine were inhibited by a novel and potent inhibitor of c-Kit tyrosine kinase [46, 47]. In this study, LY-294002 or PKC blocker (calphostin C) did not affect H_2O_2-induced effects. Generally PKC activation produces smooth muscle contraction. Therefore, these results suggest that cAMP, PI3-kinase and PKC are not involved in the H_2O_2-signal pathways, indicating that other mechanisms may be involved in the activation of K_{ATP} channels in intestinal ICC by H_2O_2.

H_2O_2 increases tyrosine phosphorylation of proteins in different cell types [32]. Receptor tyrosine kinase, such as platelet-derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR) and insulin-like growth factor (IGFR), have been

demonstrated to be targets of ROS [48, 49]. It has been reported that H_2O_2-induced p44/42 activation was mediated by the receptor tyrosine kinase of EGFR in feline ileal smooth muscle cells and vascular smooth muscle cells [32, 33]. Based on these findings, we examined the activation of EGFR using an EGFR antagonist (AG1478) before exposure to H_2O_2. We found that EGFR antagonist inhibited the H_2O_2-induced effects. It has been reported that PGE_2 induced EGRF transactivation, leading to the promotion of gastrointestinal cancer cell growth [35]. Furthermore, the PGE_2 receptor EP4 agonist promotes hepatocytes proliferation through EGFR and ERK phosphorylation [50]. In the present study, an EGFR receptor tyrosine kinase inhibitor inhibited PGE_2-induced effects. Taken together these findings suggest that the activation of EGFR by H_2O_2 *via* PGE_2 may involve the regulation of pacemaker currents.

In conclusion, we have demonstrated that the inhibition of pacemaker currents by H_2O_2 in cultured ICC has mediated the activation of COX, with a consequent increase of PGE_2 production. And we have showed that during these events, the increasing of PGE_2 can influence on K_{ATP} channels, the receptor tyrosine kinase of EGFR and also MAPK (p44/42) pathway.

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