Korean J Physiol Pharmacol
Vol 11: 15–20, February, 2007

Inhibition of Pacemaker Activity of Interstitial Cells of Cajal by Hydrogen Peroxide via Activating ATP-sensitive K^+ Channels

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To investigate whether hydrogen peroxide (H_2O_2) affects intestinal motility, pacemaker currents and membrane potential were recorded in cultured interstitial cells of Cajal (ICC) from murine small intestine by using a whole-cell patch clamp. In whole cell patch technique at 30°C, ICC generated spontaneous pacemaker potential under current clamp mode (I=0) and inward currents (pacemaker currents) under voltage clamp mode at a holding potential of −70 mV. When ICC were treated with H_2O_2 in ICC, H_2O_2 hyperpolarized the membrane potential under currents clamp mode and decreased both the frequency and amplitude of pacemaker currents and increased the resting currents in outward direction under voltage clamp mode. Also, H_2O_2 inhibited the pacemaker currents in a dose-dependent manner. Because the properties of H_2O_2 action on pacemaker currents were same as the effects of pinacidil (ATP-sensitive K^+ channels opener), we tested the effects of glibenclamide (ATP-sensitive K^+ channels blocker) on H_2O_2 action in ICC, and found that the effects of H_2O_2 on pacemaker currents were blocked by co- or pre- treatment of glibenclamide. These results suggest that H_2O_2 inhibits pacemaker currents of ICC by activating ATP-sensitive K^+ channels.

Key Words: Hydrogen peroxide, Interstitial cells of Cajal, Pacemaker currents, ATP-sensitive K^+ channels

INTRODUCTION

Hydrogen peroxide (H_2O_2) generated during normal cellular metabolism is widespread, and may evoke the destruction of many cell types through necrotic or apoptotic process when present in excess (Olanow, 1992; Castagne et al, 1999).

Many studies suggested that H_2O_2 has an important role in gastrointestinal (GI) inflammation process: H_2O_2 production is increased in the mucosa of patients with ulcerative colitis as well as in animal models of inflammation (Kesharzian et al, 1992; Simmonds et al, 1992; Grisham, 1994). In addition, it is well known that inflammation suppresses GI motility. The experimental colitis showed colonic dysmotility in rats (Morteau et al, 1993) and acute experimental colitis decreases colonic circular smooth muscle contractility in rats and dogs (Sethi & Sarna, 1991; Myers et al, 1997). It is thought that these alterations in GI motility are due to the production of H_2O_2, reactive cytokines, and lipid mediators, etc. Especially, many studies showed the action of H_2O_2 on GI motility during inflammation process. H_2O_2 decreases smooth muscle contractility in ulcerative colitis patients (Cao et al, 2004) and increases the tension of ileal smooth muscle strips, whereas it inhibites the contractility of such strips after electrical stimulation (Moummi et al, 1991).

Interstitial cells of Cajal (ICC) generate electrical pacemaker activity in GI muscles (Ward et al, 1994; Sanders, 1996). The electrical activity propagates with ICC networks, conducts into smooth muscle cells via gap juctions, and intiates phasic contractions via activation of Ca^{2+} entry through L-type Ca^{2+} channels. In particular, a recent report suggests that deoxycholic acid inhibits pacemaker currents by activating ATP-dependent K^+ channels through PGE_2 in ICC of the murine small intestine (Jun et al, 2005).

Although previous studies have shown that H_2O_2 influences motility in the small intestine, the action of H_2O_2 on pacemaker activity of ICC has not yet been examined. In this study, therefore, we investigated the possibility that H_2O_2 affects the electrical properties of cultured ICC from murine small intestine by activating ATP-dependent K^+ channels.

METHODS

All experiments were carried out according to the guiding principles for the care and use of animals approved by the ethics committee in Chosun University and the National

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ABBREVIATIONS: ICC, unterstitial cells of cajal; H_2O_2, hydrogen peroxide.

Institutes of Health Guide for the Care and Use of Laboratory Animals. Every effort was made to minimize both the number of animals used and their suffering.

Material

Glibenclamide and pinacidil were purchased from RBI (Natick, NA, USA); hydrogen peroxide from sigma (St. Louis, MO, USA). For stock solutions, all drugs were dissolved in distilled water or dimethylsulfoxide and stored at −20°C.

Preparation of cells and tissues

Balb/C mice (8~13 days old) of either sex were anesthetized with ether and sacrificed by cervical dislocation. Small intestines from 1 cm below the pyloric ring to the cecum were removed and opened along the mesenteric border. Luminal contents were removed by washing with Krebs-Ringer bicarbonate solution, tissues were pinned to the base of a Sylgard dish, and mucosa was removed by sharp dissection. Small tissue stripes of intestinal muscle (contained both circular and longitudinal muscles) were equilibrated in Ca^{2+}-free Hank's solution (containing in mM: KCl 5.36, NaCl 125, NaOH 0.34, Na_2HCO_3 0.44, glucose 10, sucrose 2.9 and HEPES 11) for 30 min. The cells were then dispersed in an enzyme solution containing collagenase (Worthington Biochemical Co, Lakewood, NJ, USA) 1.3 mg/ml, bovine serum albumin (Sigma) 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 a 35 mm culture dish, and 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 murine stem cell factor (SCF, 5 ng/ml, Sigma).

Patch clamp experiments

The whole-cell configuration patch-clamp technique was used to record cultured ICC membrane currents (voltage clamp) and potentials (current clamp), and Axopatch 1-D (Axon Instruments, Foster, CA, USA), and membrane currents and potentials were amplified. Command pulses were applied using an IBM-compatible personal computer and pClamp software (version 7.2; Axon Instruments). Data were filtered at 5 kHz and displayed on an oscilloscope, a computer monitor, and a pen recorder (Gould 2200, Gould, Valley View, OF, USA). The cells were bathed in a solution containing (in mM): KCl 5, NaCl 135, $CaCl_2$ 2, glucose 10, $MgCl_2$ 1.2 and HEPES 10, adjusted to pH 7.4 with Tris. The pipette solution contained (in mM) KCl 140, $MgCl_2$ 5, K_2ATP 2.7, Na_2GTP 0.1, creatine phosphate disodium 2.5, HEPES 5 and EGTA 0.1, adjusted to pH 7.2 with Tris. Results were analyzed using pClamp and Graph Pad Prism (version 2.01) software. All experiments were performed at 30°C.

Statistical analysis

Data were expressed as mean±standard errors. Differences were evaluated using the Student's t test. P values of <0.05 were taken to be statistically significant. The n values reported in the text refer to the number of cells used in patch-clamp experiments.

RESULTS

Spontaneous activity in interstitial cells of Cajal

ICC cultured from the murine small intestine were identified with the Kit immunofluroescence. Cells with Kit positive had a distinctive morphology that was easily recognized in cultures (Jun et al, 2005). Recording from cultured ICC showed spontaneously pacemaker potentials under current clamp mode (I=0). The resting membrane potential was −47±5 mV, and amplitude was 28±7 mV (n=4, Fig. 1A). Converting the amplifier to voltage clamp mode at a holding potential −70 mV, ICC generated spontaneous inward currents called 'pacemaker currents'. The average frequency of the currents was 13±1 cycles/min, and the amplitude were averaged −529±39 pA (n=4, Fig. 1B).

Effects of H_2O_2 on membrane potentials and the pacemaker currents in interstitial cells of Cajal

We tested the effects of H_2O_2 on ICC under current and voltage clamp mode. Under current clamp condition, H_2O_2 (100 μM) treatment induced hyperpolarization of resting membrane potential and decreased the amplitude of the potentials (Fig. 2A). The frequency and amplitude of pacemaker currents were decreased under voltage clamp mode at a holding potential of −70 mV and the resting currents increased in the outward direction, when treated with H_2O_2 (100 μM) (Fig. 2B). Taken together, these results suggest that H_2O_2 may have a function in ICC.

Dose dependent effects of H_2O_2 on pacemaker current in interstitial cells of Cajal

Under voltage clamp mode at a holding potential −70 mV, the addition of H_2O_2 (100 μM) inhibited the frequency and amplitude of pacemaker currents in ICC, shown in Fig. 2. To find whether the effects of H_2O_2 on pacemaker currents in ICC is dose-dependent or not, we treated ICC with

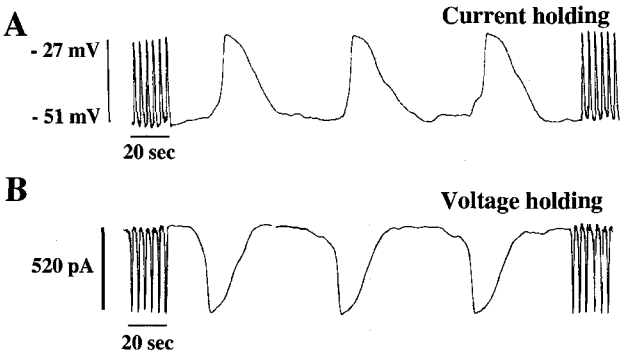

Fig. 1. Typical trace of pacemaker potentials in current clamping mode (I=0) (A) and spontaneous pacemaker currents in voltage clamping mode recorded at a holding potential of −70 mV (B) in ICC.

various concentration of H_2O_2. Under voltage clamp mode at a holding potential of -70 mV, ICC showed spontaneous inward currents (pacemaker currents); The mean frequency of these pacemaker currents was 13 ± 1.4 cycles/min and their mean amplitude and mean resting current level were -480 ± 52 pA and -27 ± 4 pA, respectively (n=4). The addition of 10 μM H_2O_2 reduced the amplitude and frequency of the pacemaker currents slightly (Fig. 3A); frequencies were 12.2 ± 1.7 cycles/min, and the resting currents and amplitudes were 12.5 ± 6 pA and -320 ± 12 pA respectively (Fig. 3D~F). When treated with 100 μM and 1 mM H_2O_2 under voltage clamp condition, pacemaker currents in ICC were largely inhibited and resting currents were increased in the outward direction (Fig. 3B & C). The inhibitory frequencies and amplitudes were 5.6 ± 2.8 cycles/ min and -72.6 ± 16 pA at 100 μM H_2O_2 and 2.9 ± 2.1 cycles/ min and -30.4 ± 10 pA at 1 mM H_2O_2, respectively. The resting current levels were 21.2 ± 2 pA at 100 μM H_2O_2 and 25 ± 2.1 pA at 1 mM H_2O_2 (n=4) (Fig. 3D~F). These data indicate that H_2O_2 dose-dependently inhibits pacemaker currents in ICC.

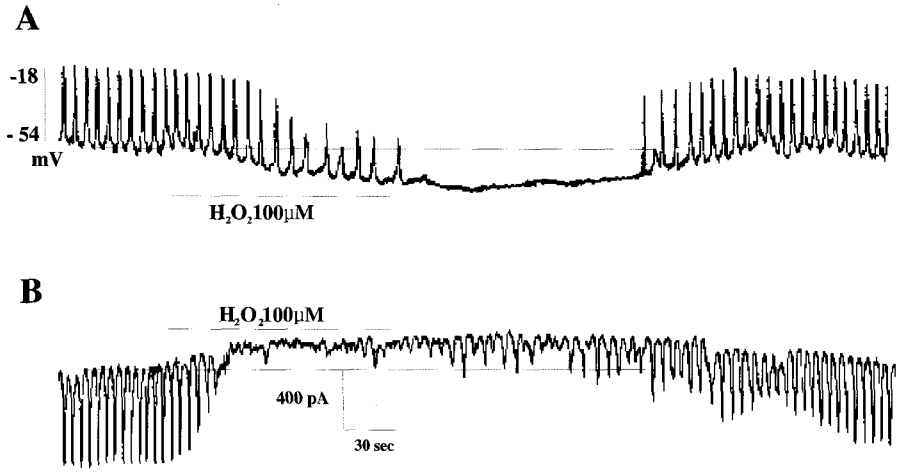

Fig. 2. Effects of H_2O_2 on pacemaker potentials and pacemaker currents recorded in cultured ICC. (A) shows the pacemaker potentials of ICC exposed to H_2O_2 (100 μM) in the current clamping mode (I=0). H_2O_2 induced membrane hyperpolarization. (B) shows the pacemaker currents of ICC exposed to H_2O_2 (100 μM) recorded at a holding potential of -70 mV. The frequency and amplitude of pacemaker currents were decreased, and the resting currents were increased in the outward direction by H_2O_2. Dotted lines indicate zero current levels.

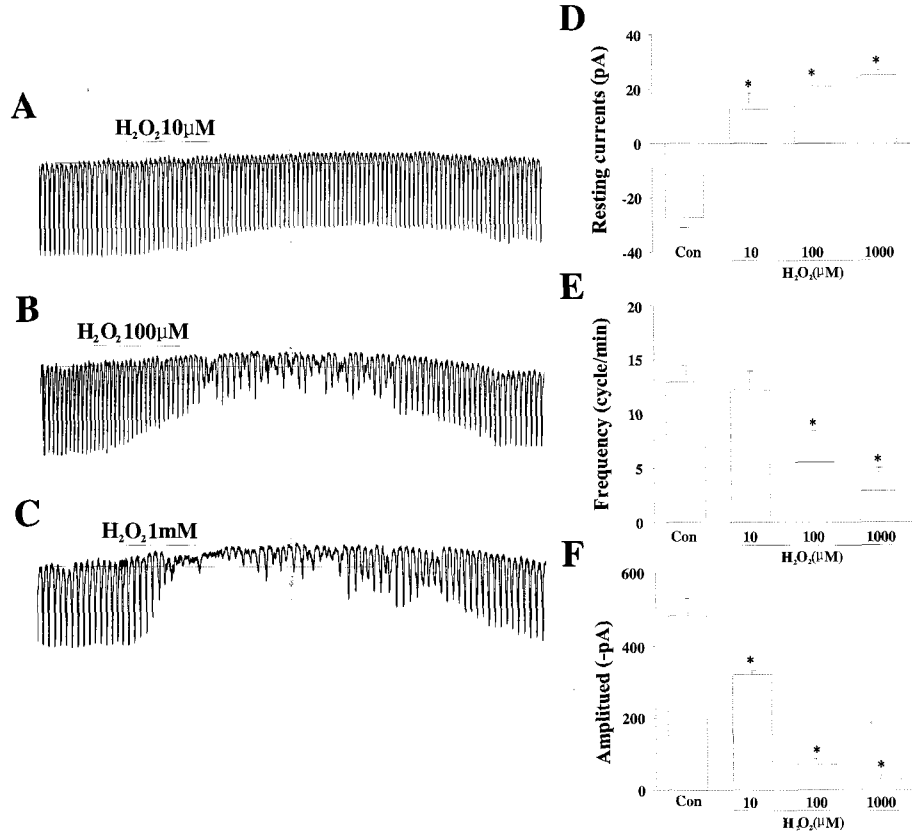

Fig. 3. The dose-dependent effects of H_2O_2 on pacemaker currents in cultured ICC (A~C) show pacemaker currents of ICC exposed to H_2O_2 (10, 100 μM, and 1 mM) at a holding potential of -70 mV. H_2O_2 inhibited spontaneous pacemaker currents in a dose-dependent manner in ICC and increased resting currents in the outward directions. (D~F) Summarize the inhibitory effects of H_2O_2 on pacemaker currents in ICC. Bars represent mean$\pm$SE (n=4/ group). *Asterisks mean significantly different from the controls ($p<0.05$), and dotted lines indicate zero current levels. Con: control.

Involvement of ATP-sensitive K^+ channels in the effects of H_2O_2 on pacemaker currents in interstitial cells of Cajal

In order to elucidate whether H_2O_2 affects ATP-sensitive K^+ channels in ICC, we first investigated the effects of pinacidil, ATP-sensitive K^+ channels opener, and glibenclamide (ATP-sensitive K^+ channels blocker) on pacemaker currents. As shown in Fig. 4A, under current clamp mode, pinacidil (10 μM) caused hyperpolarization of resting membrane potential and decreased the amplitude of the potentials in ICC, and these effects were recovered by co-treatment with glibenclamide (10 μM). Futhermore, under voltage clamp mode at a holding potential -70 mV, pinacidil (10 μM) decreased the frequency and amplitude of the pacemaker currents, and also increased the resting currents in the outward direction. The pinacidil-induced alterations returned to the control levels by co-treating with glibenclamide (10 μM) (Fig. 4B). These effects of pinacidil were analogous to those of H_2O_2.

For more verification, we tested the effects of H_2O_2 on pacemaker currents under co- or pre-treatment of glibenclamide in ICC. In voltage clamp mode at a holding potential -70 mV, we confirmed again that the frequency and the amplitude of pacemaker currents were decreased and

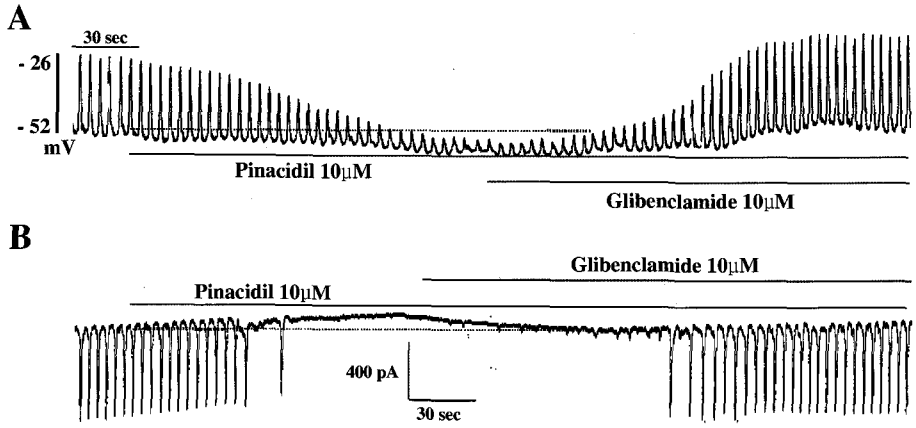

Fig. 4. Effects of pinacidil on pacemaker currents in ICC of the murine small intestine. (A) shows the slow waves of ICC exposed to pinacidil (10 μM) in the currents clamping mode (I=0). Pinacidil-induced membrane hyperpolarization and decreased amplitude of pacemaker potentials were reversed by glibenclamide (10 μM). (B) shows the pacemaker currents of ICC exposed to pinacidil (10 μM) at a holding potential of -70 mV. Pinacidil decreased the frequency and amplitude of the pacemaker currents, and increased the basal outward currents, and these effects were reversed by adding glibenclamide (10 μM). Dotted lines indicate zero current levels.

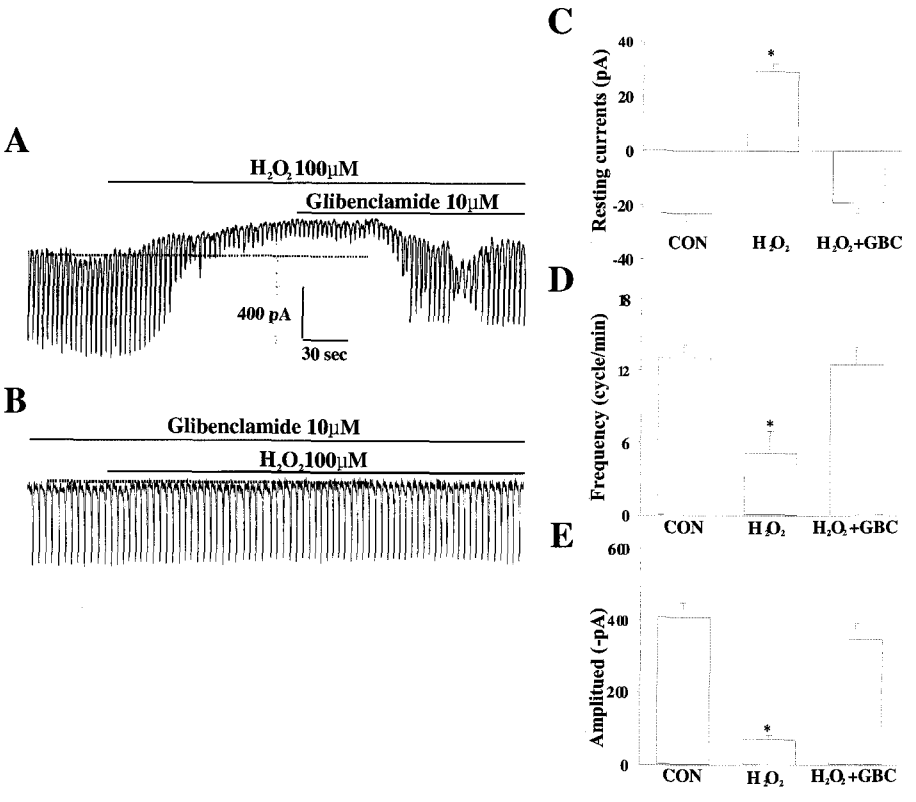

Fig. 5. Glibenclamide blockade of H_2O_2 induced attenuation of pacemaker currents in cultured murine small intestine ICC. (A) shows the pacemaker currents exposed to H_2O_2 (100 μM) at a holding potential of -70 mV. H_2O_2 decreased the frequency and the amplitude of pacemaker currents, and increased basal outward currents. These effects were reversed by adding glibenclamide (10 μM). The blocking response to glibenclamide is summarized in (C~E). Bars represent mean values ± SE (n=4). *Asterisks mean significantly different from the controls ($p<0.05$). (B) shows the effect of H2O2 on pacemaker currents after pretreatment of cells with glibenclamide. Dotted lines indicate zero current levels. Con: control, GBC: glibenclamide.

the resting currents were increased in outward direction, when ICC were treated with H_2O_2 (100 μM). Furthermore, co-treatment of glibenclamide (10 μM) with H_2O_2 returned the decreased frequency (control vs H_2O_2=13±1.1 cycles/min vs 5.1±1.8 cycles/min) and amplitude (control vs H_2O_2 =−409±39 pA vs −69±14 pA) of pacemaker currents to the control levels (the frequency: 12.4±1.5 cycles/min and the amplitude: −347±48 pA). The resting currents increased by H_2O_2 (control vs H_2O_2=−23±3 pA vs 29±3 pA) also returned to the base line by glibenclamide treatments (the resting currents: −19±4 pA) (n=4, Fig. 5A, C~E). Furthermore, pretreatment of ICC with glibenclamide (10 μM) almostly blocked the effects of H_2O_2 on pacemaker currents (n=4, Fig. 5B). These results indicate that the inhibitory effects of H_2O_2 on pacemaker currents in ICC maybemediated via ATP-sensitive K^+ channels.

DISCUSSION

The present study was undertaken to examine the inhibitory action of H_2O_2 on spontaneous electrical activity in cultured ICC. The inhibitory action of H_2O_2 was found to be mediated through the activation of ATP-sensitive K^+ channels.

Previous reports have shown that ICC generate spontaneous pacemaker currents that depolarize membrane, which then spreads to smooth muscle via gap junctions, resulting in the depolarization of smooth muscle cell membrane (Koh et al, 1998; Thomsen et al, 1998). In our study, the cultured ICC showed spontaneous depolarization under current clamp mode (Fig. 1A) and also this activity was mirrored by spontaneous inward currents under voltage clamp mode at a holding potential −70 mV (Fig. 1B). Thus, these results indicated that our cultured ICC are useful in investigation of the electrical rhythm and frequency regulation of pacemaker potentials and pacemaker currents in small intestine.

Many reports showed that chronic and tonic inflammatory conditions in GI tract contribute to motor dysfunction. Furthermore, H_2O_2 production has been shown to increase in the muscularis during inflammation, which may be a contributing factor to the suppression of contraction seen in inflammation, and incubation of normal muscle strips with H_2O_2 suppresses contractions and generation of tone. These effects are similar to those seen in patients with inflammation in small intestine (Kern et al, 1951; Chaudhary & Truelove, 1961; Sethi & Sarna, 1991). These results suggest that H_2O_2 may be one of the inflammatory response mediators that contribute to motility defects and digestive dysfunction in acute and chronic inflammation in GI tract. In this study, H_2O_2 inhibited the pacemaker currents and decreased the amplitude of the pacemaker potentials in ICC (Fig. 2). In terms of its concentration, H_2O_2 was found to have dose-dependent inhibitory effects on pacemaker currents in ICC (Fig. 3). Therefore, these results suggest that H_2O_2 can alter GI motility by acting on ICC during inflammation. The regulation of pacemaker potentials or pacemaker currents in ICC is important, because their impairment is likely to cause various motility disorders.

Many studies found that ATP-sensitive K^+ channels play important roles in regulating resting membrane potential and membrane excitability in a variety of tissues. It is well known that ATP-sensitive K^+ channels are inhibited by glibenclamide, a selective ATP-sensitive K^+ channels blocker, and activated by ATP-sensitive K^+ channels agonists (pinacidil, cromakalim, and diazoxide) (Bray & Quast, 1992; Edwards & Weston, 1993; Kitamura & Kuriyama, 1994; Teramoto & Brading, 1996). Especially, a recent report suggested that ATP-dependent K^+ channels regulate pacemaker currents in ICC, and that they are comprised of K_{ir} 6.2 and SUR 2B subunits in ICC (Choi et al, 2006). Moreover, some reports suggested that H_2O_2 directly modulates K^+ channels by oxidizing sulfhydryl groups of channel proteins (Ruppersberg et al, 1991; Vega-Saenz de Miera & Rudy, 1992; Bannister et al, 1999). These channels include votage-dependent K^+ channels (Ruppersberg et al, 1991; Vega-Saenz de Miera & Rudy, 1992), inward-rectifier K^+ channels (Bannister et al, 1999), ATP-sensitive K^+ channels (Weik & Neumcke, 1986; Islam et al, 1993), and Ca^{2+} -activated K^+ channels (Wang et al, 1997). In our study, pinacidil were found to inhibit pacemaker currents, and activate outward currents, which were antagonized by glibenclamide (Fig. 4A), indicting that ATP-sensitive K^+ channels exist in ICC, and that the activity of ATP- sensitive K^+ channels in ICC may be involved in intestinal motility. Because the effects of pinacidil on pacemaker currents were similar in nature to the inhibitory action of H_2O_2 in ICC, we also examined whether glibenclamide inhibited the effects of H_2O_2 on pacemaker currents in ICC. As shown in Fig. 5, we observed that the inhibitory effects of H_2O_2 recovered by application of glibenclamide. These results suggested that H_2O_2 suppresses intestinal motility by activating ATP-sensitive K^+ channels in ICC of murine small intestine.

In summary, the present results indicate that H_2O_2 directly alters pacemaker currents by modulating ATP-sensitive K^+ channels, thus suggesting a possibility that GI motor dysfunction induced by H_2O_2 during inflammatory process may involve the regulation of ICC by H_2O_2. However, more studies are required for elucidation of the signaling pathway involved in the inhibitory action of H_2O_2 in ICC.

ACKNOWLEDGEMENT

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2005-041-E00031).

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