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PGE$_2$ Regulates Pacemaker Currents through EP$_2$-Receptor in Cultured Interstitial Cells of Cajal from Murine Small Intestine

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The interstitial cells of Cajal (ICCs) are the pacemaker cells in gastrointestinal tract and generate electrical rhythmicity in gastrointestinal muscles. Therefore, ICC may be modulated by endogenous agents such as neurotransmitter, hormones, and prostaglandins (PGs). In the present study, we investigated the effects of prostaglandins, especially PGE$_2$, on pacemaker currents in cultured ICCs from murine small intestine by using whole-cell patch clamp techniques. ICCs generated spontaneous slow waves under voltage-clamp conditions and showed a mean amplitude of -452 ± 39 pA and frequency of 18 ± 2 cycles/min (n=6). Treatments of the cells with PGE$_2$ (1 μM) decreased both the frequency and amplitude of the pacemaker currents and increased the resting currents in the outward direction. PGE$_2$ had only inhibitory effects on pacemaker currents and this inhibitory effect was dose-dependent. For characterization of specific membrane EP receptor subtypes, involved in the effects of PGE$_2$ on pacemaker currents in ICCs, EP receptor agonists were used: Butaprost (1 μM), EP$_2$ receptor agonist, reduced the spontaneous inward current frequency and amplitude in cultured ICCs (n=5). However sulprostone (1 μM), a mixed EP$_1$ and EP$_3$ agonist, had no effects on the frequency, amplitude and resting currents of pacemaker currents (n=5). SQ-22536 (an inhibitor of adenylate cyclase; 100 μM) and ODQ (an inhibitor of guanylate cyclase; 100 μM) had no effects on PGE$_2$ actions of pacemaker currents. These observations indicate that PGE$_2$ alter directly the pacemaker currents in ICCs, and that the PGE$_2$ receptor subtypes involved are the EP$_2$ receptor, independent of cyclic AMP- and GMP-dependent pathway.

Key Words: Prostaglandin E$_2$, Interstitial cells of Cajal (ICCs), Pacemaker currents, EP$_2$ receptor

INTRODUCTION

Prostaglandins (PGs) are widely distributed throughout the gastrointestinal tract and play a significant role in the physiology and pathophysiology (Ahlquist et al, 1982; Wallace et al, 1984; Whittle and Vane, 1987). Many reports indicate that PGs affect water and electrolyte transport, mucous secretion and blood flow (Robert, 1991). Especially, PGs act as local regulatory agents, controlling smooth muscle contractile activity at different levels of the digestive tract, in particular the small intestine (Bueno et al, 1985; Sanders, 1984; Staumont et al, 1990). This action varies greatly, depending on the concentration, the organ, the species and even the muscle layer studied (Eglen and Whiting, 1988; Gardiner, 1986; Staumont et al, 1990). In general, PGE2 is well known to contract the longitudinal muscle and to relax the circular one in human and various animal species (Sanders, 1981; Gardiner, 1986).

Previous studies demonstrated that PGE$_2$ exerts its biological actions through binding to four specific membrane receptor subtypes, known as EP$_1$, EP$_2$, EP$_3$ and EP$_4$ (Coleman et al, 1985; Coleman et al, 1987a). These subdivisions are based on the relative potency of selective agonists and antagonists in both functional and binding studies. To date, ilprost has been known to be a more selective agonist for EP$_1$ receptor (Sheldrick et al, 1988), butaprost the most selective agonist for the EP$_2$ receptor (Gardiner, 1990), and sulprostone active on both the EP$_1$ and EP$_3$ receptors (Schaaf et al, 1981). Currently, the most recently identified EP$_4$ receptor is not known to have any selective agonists.

Many regions of the tunica muscularis of the gastrointestinal tract display spontaneous contraction, and these spontaneous contractions are mediated by periodic generation of electrical slow waves (Szurszewski, 1987). Recent studies have shown that the interstitial cells of Cajal (ICCs) act as pacemakers and conductors of electrical slow waves in gastrointestinal smooth muscles (Langton et al, 1989; Ward et al, 1994; Huizinga et al, 1995; Sanders, 1996; Ordog et al, 1999). Although the exact mechanisms for these events remain still unclear, several studies suggest that endogenous agents such as neurotransmitter, hormones and paracrine substances modulate gastrointestinal

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ABBREVIATIONS: ICCs, interstitial cells of Cajal; PGs, prostaglandins; PGE$_2$, prostaglandin E$_2$; TXA$_2$, Thromboxane A$_2$; AMP, Adenosine monophosphate; GMP, Guanosine monophosphate tract motility by influencing ICCs.

Previous studies have shown that PGE_2 influences motility in small intestine (Sanders, 1984; Bueno et al, 1985; Staumont et al, 1990). In this study, therefore, we investigated the possibility that PGE_2 might affect electrical properties of cultured ICC cells. In addition, EP receptor subtypes, involved in these effects, were also characterized.

METHODS

Material

SC-19220, butaprost and sulprostone were purchased from Cayman Chemicals. glibenclamide from Calbiochem Co., and prostaglandin E_2 from Sigma Chemical Co. For stock solutions, all drugs were dissolved in distilled water (DW) or dimethylsulfoxide (DMSO) and stored at −20°C.

Preparation of cells and tissues

Balb/C mice (8~13 days old) of either sex were anethetized with ether and sacrificed by cervical dislocation. The small intestines from 1 cm below the pyloric ring to the cecum were removed and opened along the mesenteric border. Luminal contents were washed away with Krebs-Ringer bicarbonate solution and the tissues were pinned to the base of Sylgard dish and the mucosa was removed by sharp dissection. Small tissue stripes of intestinal muscle (contained both circular and longitudinal muscles) were equilibrated for 30 min in Ca^{2+}-free Hanks solution containing 5.36 mM KCl, 125 mM NaCl, 0.34 mM NaOH, 0.44 mM Na_2HCO_3, 10 mM glucose, 2.9 mM sucrose and 11 mM HEPES. Then, 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 dish. 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 murine stem cell factor (SCF, 5 ng/ml, sigma). Interstitial cells of Cajal (ICCs) were indentified immunologically with a monoclonal antibody for Kit protein (ACK_2) labelled with Alexa Fluor 488 (molecular prove, Eugene, OR, USA) (Koh et al, 1998; Koh et al, 2000). Morphologies of ICCs are distinct from other cell types in the culture, therefore it was possible to identify the cells with phase contrast microscopy, when the cells were once verified with ACK2-Alexa Fluor 488 labeling.

Patch clamp experiments

The whole-cell configuration of the patch-clamp technique was used to record membrane currents (voltage clamp) and potentials (current clamp) in cultured ICCs, and Axopatch 1-D (Axon Instruments, Foster, CA, USA) amplified membrane currents and potentials. Command pulse was applied using IBM-compatible personal computer and pClamp software (version 6.1; Axon Instruments). The data were filtered at 5 kHz and displayed on an oscilloscope, a computer monitor and a pen recorder (Gould 2200, Gould, Vally view, OF, USA). 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. 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 means ± standard errors. Differences in the data were evaluated by 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 patch-clamp experiments.

RESULTS

Spontaneous inward currents and depolarizations in ICCs

Under a voltage clamp at a holding potential of −70 mV, ICCs showed spontaneous inward currents, which is referred to as pacemaker current (Fig. 1A). The frequency of the pacemaker currents was 14 ± 1.6 cycles/min and the amplitude and resting current level were −420 ± 57 pA and −22 ± 18 pA, respectively (n=8; bar graph not shown). Converting the amplifier to current clamp mode, spontaneous depolarization was generated in ICCs (Fig. 1B). In the remainder of the experiments, we used a constant holding potential of −70 mV.

Effect of PGE_2 on pacemaker currents in cultured ICCs

Previous reports suggested that naturally occurring prostaglandins (PGs) comprise PGs D_2, E_2, $F_{2\alpha}$, I_2 and TXA_2 (Kennedy et al, 1982; Coleman et al, 1984). Because of their diverse action on gastrointestinal tract, therefore we chose

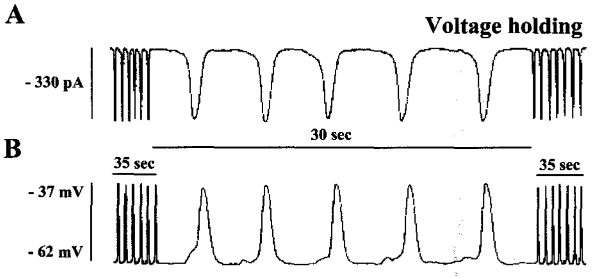

Fig. 1. Spontaneous inward currents and depolarizations in cultured ICCs of the murine small intestine. (A) Under a voltage clamp at a holding potential of −70 mV, ICCs showed spontaneous inward currents oscillations, called pacemaker currents. (B) Under a currents clamp mode, spontaneous depolarization was generated from the same cell.

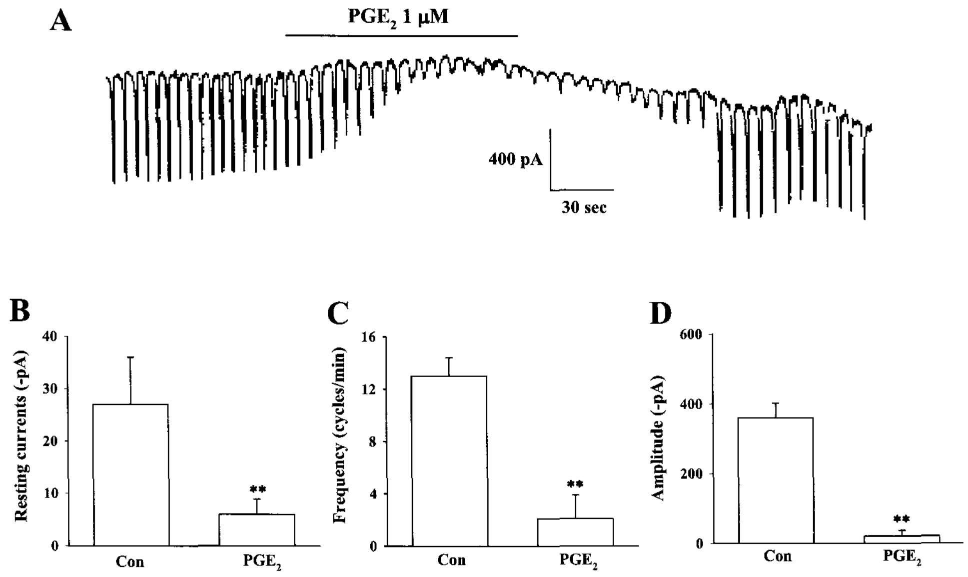

Fig. 2. Effects of prostaglandins (PGs) on pacemaker currents in cultured ICCs of the murine small intestine. Under control conditions at a holding potential of −70 mV, (A) PGE2 (1 μM) inhibited the amplitude and the frequency of pacemaker currents and increased the resting currents in the outward direction in ICCs. (B), (C) and (D) summarize the inhibitory effects of PGE2 on pacemaker currents. Each bar represents mean±SE. (n=9). Those noted with *were significantly different from the control (p<0.05).

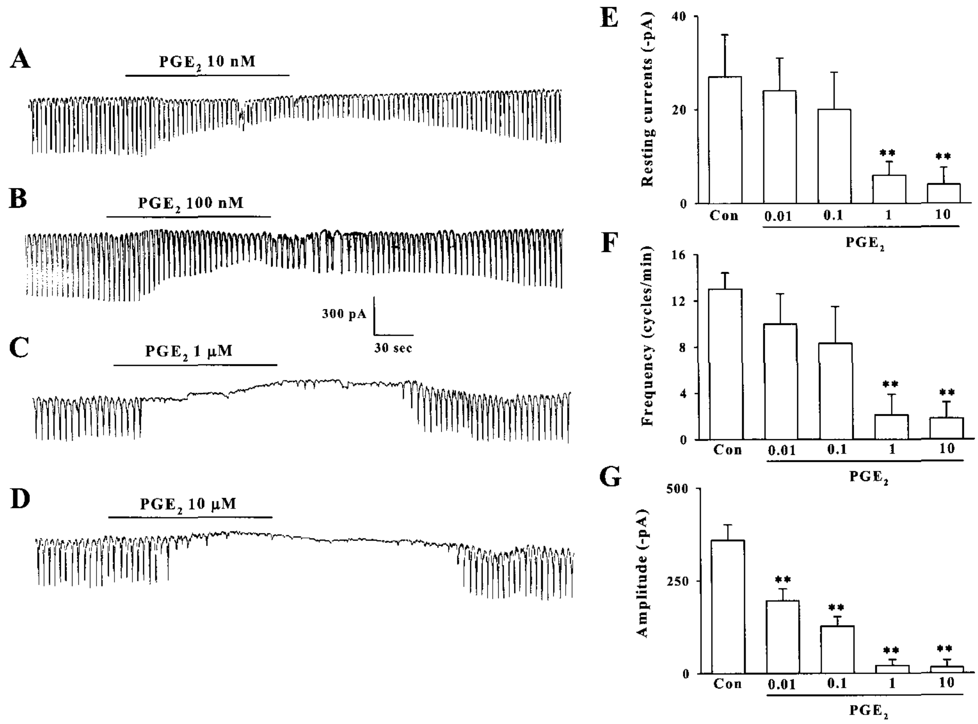

Fig. 3. Dose-dependent effects of PGE2 on pacemaker currents in cultured ICCs of the murine small intestine. (A), (B), (C) and (D) show the slow waves of ICCs exposed to PGE2 (0.01, 0.1, 1 and 10 μM) at a holding potential of −70 mV. PGE2 inhibited spontaneous pacemaker currents in dose-dependent manner in ICCs and showed increased resting currents in the outward directions. (E), (F) and (G) summarize the inhibitory effects of PGE2 on pacemaker currents in ICCs. Each bar represents mean±SE. (n=6~7/group). Those noted with *were significantly different from the controls (p<0.05).

PGE_2 to examine the effects on pacemaker currents in cultured ICCs. Under control conditions at a holding potential of −70 mV, the frequency, the amplitude and resting current level were 15±1.8 cycles/min, −418±39 pA and −24±12 pA, respectively. When PGE_2 (1 μM) was applied in ICCs, both the frequency and the amplitude of pacemaker currents were decreased, and the resting currents were increased in the outward direction under voltage-clamp conditions (−10±15 pA) (Fig. 2A). Also, the corresponding frequencies and amplitude were 3.2±0.8 cycles/min and −26.4±28 pA (Fig. 2B, C and D; n=9), respectively. These results indicate that PGE2 inhibited the frequency and amplitude of pacemaker currents.

Dose-dependency of PGE_2 actions on pacemaker currents in cultured ICCs

In previous studies, we found that PGE_2 (1 mM) have inhibitory effects on pacemaker currents in cultured ICCs. In the present study, we tested that whether PGE2 have dose-dependent or not inhibitory effects on pacemaker currents in cultured ICCs. Under a voltage clamp at a holding potential of −70 mV, ICCs generated spontaneous inward currents. The frequency of the pacemaker currents was 13±1.4 cycles/min and the amplitude and resting current level were −360±42 pA and −27±9 pA, respectively (n=6). The addition of 10 and 100 nM PGE2 slightly decreased the amplitude and the frequency of pacemaker currents in ICCs; The frequencies were 10±2.6 cycles/min at 10 nM and 8.3±3.2 cycles/min at 100 nM, and the resting currents and amplitudes were −24±7 pA and −196±32 pA at 10 nM and −20±8 pA and −127±26 pA at 100 nM (n=7; Fig. 3E, F and G), respectively. The presence of 10 and 100 nM PGE2 slightly increased resting currents in the outward direction (Fig. 3A and B). In the presence of 1 and 10 μM PGE2 under voltage-clamp condition, pacemaker currents were largely inhibited and the resting currents were also increased in outward direction (Fig. 3C and D); The inhibitory frequencies and amplitudes by PGE2 were 2.1±1.8 cycles/min and −20.9±16 pA at 1 μM PGE2 and 1.8±1.4 cycles/min and −16±19 pA at 10 μM PGE2, respectively. The resting current levels were −6±2.9 pA at 1 μM PGE_2 and −4±3.6 pA at 10 μM PGE2 (n=7; Fig. 3E, F and G). These results suggest that PGE2 inhibit pacemaker currents in dose-dependent manner in cultured ICCs.

Characterization of EP receptor subtypes, involved in the effects of PGE_2 on pacemaker currents in cultured ICCs Four subtypes of EP receptor have so far been identified, termed arbitrarily EP_1, EP_2, EP_3 and EP_4. In this study, we attempted to discern which EP receptor subtypes mediate the inhibitory actions of PGE_2 on pacemaker currents in cultured ICCs. First, we examined the effects of butaprost, a specific agonist for the EP_2 receptor subtype, on pacemaker currents in cultured ICCs. Addition of butaprost (1 μM) caused a reduction in spontaneous inward

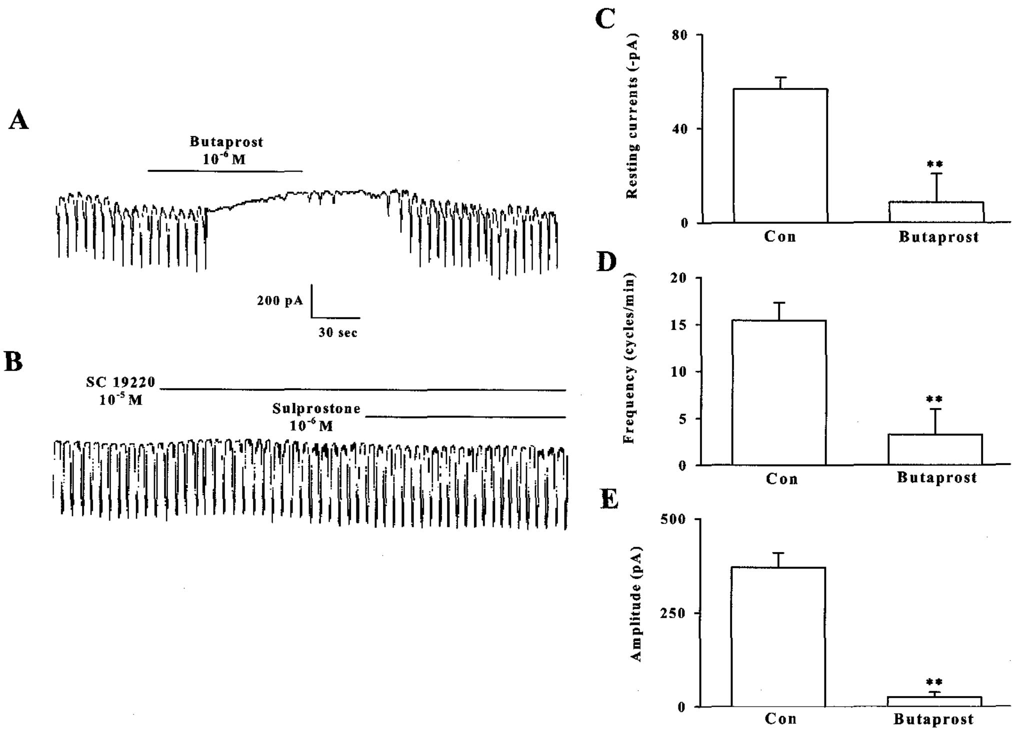

Fig. 4. Effects of EP_2 (butaprost) and EP_3 (sulprostone) receptor agonists on spontaneous inward currents from cultured ICCs. (A) Butaprost (1 μM) decreased the frequency and the amplitude of spontaneous inward current and increased the resting currents in outward directions. (B) In pretreatment with an EP_1 antagonists (SC 19220, 10 μM), sulprostone (an EP_3 and EP_1 agonists, 1 μM) had no effects on pacemaker currents. The effects of butaprost on pacemaker currents are summarized in (C), (D) and (E). Each bar represents mean±SE. (n=5/group). Those noted with * were significantly different from the controls ($p<0.05$).

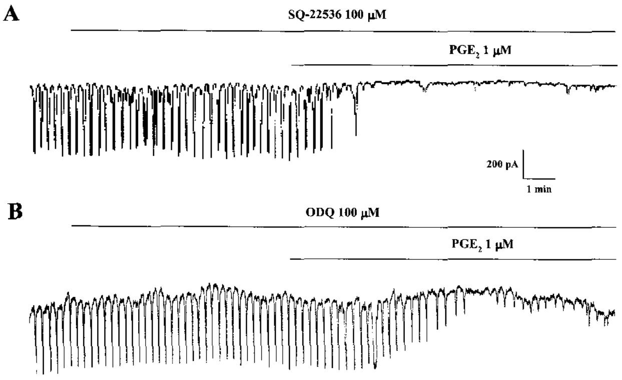

Fig. 5. Effects of SQ-22536, an inhibitor of adenylate cyclase and of ODQ, an inhibitor of guanylate cyclase, on pacemaker currents in cultured ICCs. (A) Pretreatment of SQ-22536 (100 μM) did not affect the inhibitory effects of PGE2 (1 μM) on spontaneous inward currents. (B) Pretreatment with ODQ (100 μM) also did not affect the inhibitory effects of PGE2 (1 μM) on pacemaker currents in cultured ICCs.

currents frequency and amplitude in cultured ICCs (Fig. 4A) and increased the resting currents in the outward direction (n=5; Fig. 4D, E and F) (Control vs Butaprost; The resting currents=−56±5 pA vs −8±12 pA; The amplitude=−370±39 pA vs -25±12 pA; The frequency = 15±1.9 cycles/min vs 3±2.7 cycles/min). These results are similar to those of PGE$_2$ treatments shown in previous results. Sulprostone (an EP$_3$ and EP1 receptor agonist; 1 μM) had no effects on the frequency and the amplitude of pacemaker currents in ICCs. Sulprostone also had no effects on resting currents of pacemaker currents in cultured ICCs (data not shown). In addition to the above, SC-19220, an EP1 receptor antagonist, was used in this study; Under control condition, ICCs generated spontaneous pacemaker currents, and pretreatment of ICCs with either SC-19220 (1 μM) or co-treatment with SC-19220 (1 μM) and sulprostone (1 μM) did not have any effects on pacemaker currents (Fig. 4B). These results suggest that PGE$_2$ inhibited the pacemaker currents in ICCs by stimulating EP$_2$ subtype receptors.

PGE$_2$-induced pacemaker currents inhibition was not mediated by adenylate cyclase and guanylate cyclase pathway

To investigate whether the inhibitory effects of PGE$_2$ on pacemaker currents was mediated by the cyclic nucleotide-dependent pathway, SQ-22536, an inhibitor of adenylate cyclase, and ODQ, an inhibitor of guanylate cyclase, were used. Preincubation of ICCs with SQ-22536 (100 μM) for 10 min had not effects on control states of the pacemaker currents and then co-treatment of SQ-22536 (100 μM) and PGE2 (1 μM) still inhibited the pacemaker currents (n=5; Fig. 5A), indicating that SQ-22536 had no influence on PGE$_2$-induced inhibition of the pacemaker currents. Moreover, in the presence of ODQ (100 μM), PGE$_2$ still inhibited the pacemaker currents (n=6; Fig. 5B). These results indicate that SQ-22536 or ODQ itself had no effect on pacemaker currents, and that both cyclic AMP and cyclic GMP did not mediate the inhibition of pacemaker currents by PGE$_2$.

DISCUSSION

Prostaglandins (PGs) act as local regulatory agents, controlling smooth muscle contractile activity, and PG of the E$_2$ type have been shown to contract intestinal longitudinal smooth muscle and relax circular smooth muscle (Gardiner, 1986; Sanders, 1981), implying that PGE$_2$ may regulate gastrointestinal motility. Furthermore, since ICCs generate electrical slow waves that are basic determinant of gastrointestinal motility, PGE$_2$ may have the effects on slow waves in ICCs to control gastrointestinal motility. In the present study, we demonstrated that PGE$_2$ inhibit pacemaker currents in ICCs, and characterized PGE$_2$ receptor subtypes which are involved in the inhibitory effects of PGE$_2$ on pacemaker currents.

Fatty acid cyclooxygenase in the gastrointestinal tract converts eicosatetraenoic acid (arachidonic acid) primarily to prostacyclin (prostaglandin I$_2$) and, to a lesser extent, to PGE$_2$, PGF$_{2\alpha}$ and thromboxane A$_2$ (Karim et al, 1967; Karim et al, 1968; LeDuc et al, 1979; Robert, 1981). Previous studies on motility of gastrointestinal tract showed that PGE$_2$ generally contract the longitudinal smooth muscle layer of the small intestine and relax the circular layer (Bennett et al, 1970; Waller, 1973). In contrast, PGF$_{2\alpha}$ contract both smooth muscle layers (Bennet et al, 1975). This implies that both PGE$_2$ and PGF$_{2\alpha}$ have function on motility, but different actions in gastrointestinal tract. In cultured ICCs, PGE$_2$ inhibited the frequency and the amplitude of pacemaker currents and PGE$_2$ increased the resting currents in the outward direction (Fig. 2A), suggesting that PGE$_2$ in ICCs have the inhibitory effects on pacemaker currents.

As mentioned above, PGE$_2$ have function on motility in gastrointestinal tract and this action varies, depending on species and concentration (Eglen and Whiting, 1988; Gardiner, 1986; Staumont et al, 1990). Especially, as for the concentration, PGE$_2$ have been shown to have dual effects; PGE$_2$ have suppressive effects at low concentration but, at high concentration activate colonic motility of rabbit in vivo and vitro studies (Burafoff and Percy, 1992) and stomach mechanical activity of guinea-pig (Frantzides et al, 1992). In this study, PGE$_2$ showed the inhibitory effects only on pacemaker currents in dose-dependent manner (Fig. 3) and, slight inhibitory effects or no effects at 1 nM or 100 pM (data not shown).

The recent cloning and expression of receptors for the prostaglandins (PGs) have confirmed not only the existence of at least four out of five classes of prostaglandin receptor (IP for PGI$_2$ binding, FP for PGF2a binding, EP for PGE$_2$ binding and, TP for TXA$_2$ binding), but also support the logical subdivision of EP receptors into at least three subtypes, including EP$_1$, EP$_2$ (or EP$_4$) and EP$_3$. There are also a selective agonist and antagonist for each EP receptor; To date, butaprost appears to be the most selective agonist for the EP$_2$ receptor subtype (Gardiner, 1990), and sulprostone is active on the EP1 and EP3 receptor (Schaaf et al, 1981), while currently no selective agonists for the EP$_4$ receptor subtype. SC19920 is an antagonist known to block the EP$_1$ receptor (Sanner, 1972), but as yet antagonists for EP$_2$ and EP$_3$ receptors have not been known. In this study, butaprost showed the inhibitory effects on pacemaker currents in cultured ICCs, and the pattern of butaprost effect is similar to that of PGE_2 effect (Fig. 4A). Before the addition of sulprostone, pretreatment of ICCs with SC19920 to block EP_1 receptor had no effects on spontaneous inward currents, and also co-treatment of SC19920 plus sulprostone had no effects (Fig. 4B), indicating that PGE_2 have influence on pacemaker currents in ICCs by stimulating the EP_2 receptor subtypes.

Until the late 1980s, almost all of the studies of prostaglandins (PGs) and second messengers were concerned with cyclic nucleotides, particularly cAMP. Butcher and colleagues were the first to demonstrate an association between PGs and cAMP (Butcher et al, 1967; Butcher & Baird, 1986) and, although their observation made little initial impact, it has increasingly been accepted that E-series of PGs at least are capable of stimulating adenylyl cyclase to increase intracellular cAMP (Kuehl et al, 1972, 1973). Several studies suggest the participation of cAMP in PGE2 actions, especially the EP_2 receptor. The results of Simon et al (1980) provide indirect evidence for positive coupling of an EP receptor to adenylate cyclase, however more direct evidence on the association between EP_2 receptors and cAMP generation in enterocytes has been provided by Hardcastle et al (1982). Similarly, Jumblatt and Peterson (1991) found an association between EP_2 receptor stimulation and cAMP generation in corneal endothelial cells. Furthermore, in cells expressing the recombinant murine EP_2 receptor, PGE_2 increased the intracellular cAMP level without any change in inositol phosphate content (Honda et al, 1993). These studies imply that PGE_2 may exert the actions on pacemaker currents in ICCs through cAMP signaling pathway; Namely, the generation of pacemaker currents and the its regulation in ICCs involve the cAMP signaling. However, in our recent study, treatment of ICCs with 8-bromo-cAMP (cell-permeable cAMP analog) showed no effects on the control pacemaker currents (Jun et al, 2004). Also, as shown in Fig. 5A, pretreatment with SQ-22536, an inhibitor of adenylate cyclase, did not influence the PGE_2 actions on pacemaker currents. Taken together various reports and results, PGE_2 appear to have function in diverse cells and tissues by modulating the cyclic AMP-dependent pathway, however, in ICCs PGE_2 have inhibitory actions on pacemaker currents independent of the above pathway. Further studies on PGE_2 actions in ICCs are needed, especially on second messenger.

In summary, the present results indicate that PGE_2 directly alter the pacemaker currents in ICCs. The PGE_2 receptor subtypes involved are EP_2 receptor, and the effects of PGE_2 on pacemaker currents are not mediated via cyclic AMP- and GMP-dependent pathway.

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