Activating of ATP-Dependent K⁺ Channels Comprised of K_{ir} 6.2 and SUR 2B by PGE₂ Through EP₂ Receptor in Cultured Interstitial Cells of Cajal from Murine Small Intestine

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Key Words

Prostaglandin E₂ • Interstitial cells of Cajal (ICC) • Pacemaker currents • EP₂ receptor • ATP-dependent K⁺ channels • K_{ir} 6.2, SUR 2B

Abstract

The interstitial cells of Cajal (ICC) are pacemaker cells in gastrointestinal tract and generate an electrical rhythm in gastrointestinal muscles. We investigated the possibility that PGE₂ might affect the electrical properties of cultured ICC by activating ATP-dependent K⁺ channels and, the EP receptor subtypes and the subunits of ATP-dependent K⁺ channels involved in these activities were identified. In addition, the regulation of intracellular Ca²⁺ ([Ca²⁺]_i) mobilization may be involved the action of PGE₂ on ICC. Treatments of ICC with PGE₂ inhibited electrical pacemaker activities in the same manner as pinacidil, an ATP-dependent K⁺ channel opener and PGE₂ had only a dose-dependent effect. Using RT-PCR technique, we

found that ATP-dependent K⁺ channels exist in ICC and that these are composed of K_{ir} 6.2 and SUR 2B subunits. To characterize the specific membrane EP receptor subtypes in ICC, EP receptor agonists and RT-PCR were used: Butaprost (an EP₂ receptor agonist) showed the actions on pacemaker currents in the same manner as PGE₂. However sulprostone (a mixed EP₁ and EP₃ agonist) had no effects. In addition, RT-PCR results indicated the presence of the EP₂ receptor in ICC. To investigate cAMP involvement in the effects of PGE₂ on ICCs, SQ-22536 (an inhibitor of adenylate cyclase) and cAMP assays were used. SQ-22536 did not affect the effect of PGE₂ on pacemaker currents, and PGE₂ did not stimulate cAMP production. Also, we found PGE₂ inhibited the spontaneous [Ca²⁺]_i oscillations in cultured ICC. These observations indicate that PGE₂ alters pacemaker currents by activating the ATP-dependent K⁺ channels comprised of K_{ir} 6.2-SUR 2B in ICC and this action of PGE₂ are through EP₂ receptor subtype and also the activation of ATP-dependent K⁺ channels involves intracellular Ca²⁺ mobilization.

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Introduction

Prostaglandins (PGs) are widely distributed throughout the gastrointestinal tract and play a significant role in its physiology and pathophysiology [1-3]. In particular, PGE₂ is known to contract longitudinal muscle and to relax circular muscle in humans and in various animal species [4, 5]. However, this action varies greatly, and depends on PGE₂ concentration, the organ, the species, and even the muscle layer studied [5-7]. Previous studies demonstrated that PGE₂ exerts its biological actions through binding to four specific membrane receptor subtypes known as EP₁, EP₂, EP₃ and EP₄ [8, 9]. These subdividing are the basis of the relative potency of selective agonists and antagonists in both functional and binding studies.

Recent studies have shown that the interstitial cells of Cajal (ICC) act as pacemakers and conductors of electrical slow waves in gastrointestinal smooth muscles [10-14]. Although the precise mechanisms underlying these events remain unclear, there are many evidences that spontaneous intracellular Ca²⁺ activities in ICC involve the producing of pacemaker action [15-17]. Also, several studies have suggested that endogenous agents such as neurotransmitters, hormones, and paracrine substances may modulate gastrointestinal tract motility by influencing ICC ion channels. In particular, recent reports have suggested that deoxycholic acid inhibits pacemaker currents by activating ATP-dependent K⁺ channels through PGE₂ in ICC of the murine small intestine [18]. In addition, several molecular studies have shown that functional ATP-dependent K⁺ channels are formed by a combination of a sulfonylurea receptor (SUR) and an inward rectifier K⁺ channel subunit of the K_{ir} 6 family [19-23].

Although previous studies have shown that PGE₂ influences motility in the small intestine [7, 24] and that it also modulates ATP-dependent K⁺ channels in ICC, the make up of ATP-dependent K⁺ channels, the subtypes of the PGE₂ receptor and the regulation of intracellular Ca²⁺ oscillations involved in PGE₂ action are unknown. Therefore, in this study, we investigated the possibility that PGE₂ affects the electrical properties of cultured ICC by activating ATP-dependent K⁺ channels and the action of PGE₂ involves the mobilization of intracellular Ca²⁺ oscillation in ICC. In addition, EP receptor subtypes and subunits of the ATP-dependent of K⁺ channels involved in these effects were investigated.

Materials and Methods

Materials

Butaprost, sulprostone, and SC19220 were purchased from Cayman Chemicals (Ann Arbor, MI, USA); glibenclamide from RBI (Natick, MA, USA); and prostaglandin E₂ and SQ-22536 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

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 Institutes of Health Guide for the care and Use of Laboratory Animals, and every effort was made to minimize both the number of animals used and their suffering. 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 were removed by sharp dissection. Small tissue stripes of intestinal muscle (contained both circular and longitudinal muscles) were equilibrated in Ca²⁺-free Hanks solution (containing in mM: KCl 5.36, NaCl 125, NaOH 0.34, Na₂HCO₃ 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 % O₂-5 % CO₂ 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 (ICC) were identified immunologically with a monoclonal antibody for Kit protein (ACK₂) labelled with Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA).

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) amplified membrane currents and potentials. 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, OH, USA). The cells were bathed in a solution containing (in mM): KCl 5, NaCl 135, CaCl₂ 2, glucose 10, MgCl₂ 1.2 and HEPES 10, adjusted to pH 7.4 with tris. The pipette solution contained (in mM) KCl 140, MgCl₂ 5,

K₂ATP 2.7, Na₂GTP 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.

RT-PCR with c-Kit positive cell

First, we identified the c-Kit positive cells with under a confocal laser scanning microscope and then single cell with c-Kit positive was collected by applying negative pressure to a cell in contact with recording pipette, lifting the cell out of the bath, and immediately single cell was expelled from the pipette into PCR tube, which contained lysis buffer. Total RNA was isolated from c-Kit positive single cell using a RNeasy micro kit (Qiagen, catalog no. 74004). cDNAs were produced from the total RNA using Sensiscript reverse transcriptase kit (Qiagen, catalog no. 205213). Primers used as follows: EP₁ (Accession number NM_013641), forward 5' - GCT AAG TCA AAC CAT TCT GG - 3', reverse 5' - TCT TAG AAC TAA GGT TGC GG - 3', (with a product size of 255 bp)-; EP₂ (Accession number NM_008964), forward 5' - TGT AAG GGG CTG GAA TAT AA - 3', reverse 5' - CAC ATG CTT TGT CAC TCA AC - 3', (with a product size of 225 bp)-; EP₃ (Accession number NM_011196), forward 5' - GTT AGC TGG TTC CTG TGA AG - 3', reverse 5' - GAC TTT TTG TCT TGT TTG GC - 3', (with a product size of 257 bp)-; and EP₄ (Accession number NM_008965), forward 5' - TCG TAG TAT TGT GCA AGT CG - 3', reverse 5' - TAG AAG TAG GCG TGG TTG AT - 3', (with a product size of 265 bp). K_{ir} 6.1 (Accession number D88159), forward 5' - AGA CAG TTA CTT GTT GAG TCC TG - 3', reverse 5' - ATA CAG GAA GTT TTA ATG TCT CAA AT - 3', (with a product size of 201 bp)-; K_{ir} 6.2 (Accession number D50581), forward 5' - ATC ATT GTC ATC TTG GAA GG - 3', reverse 5' - AGC TTC TTG ATG ACA GGA AA - 3', (with a product size of 340 bp)-; SUR 1 (Accession number L40624), forward 5' - CTT ACG AGA ATA TGG TAA CTG AGA T - 3', reverse 5' - TTA GAA GAT ATT CCA CAG CTC TAT C - 3', (with a product size of 113 bp), SUR 2B (Accession number D86038), forward 5' - GGG TTT CTC TGT ATA GCC CT - 3', reverse 5' - AGC TTC TTG ATG ACAGGAAA - 3', (with a product size of 347 bp), c-kit (Accession number Y00864), forward 5' - CAC TGT CCA ACA TAA AGG GT - 3', reverse 5' - GGA AAG GTG CAA GAG TGT AG - 3', (with a product size of 276 bp), myosin (Accession number NM_010860), forward 5' - GAG ATG AAT GTG AAG GTG CT - 3', reverse 5' - CTT CCT CTG TCA TCT TCT CG - 3', (with a product size of 315 bp), and PGP9.5 (Accession number AF172334), forward 5' - AAA GCAAAT TGA GGA ACT GA - 3', reverse 5' - GTT CTT CTC GAA ACA CTT GG - 3', (with a product size of 226 bp). PCR mixtures contained 1× reaction buffer, 0.2 mM deoxynucleotide triphosphates, 2.25 mM MgCl₂, each primer at 100 nM, and 1-2 µl of RT product in an Advantage cDNA polymerase mix (Clontech, Palo Alto, CA, U.S.A.). Cycling conditions; for c-kit, myosin, PGP9.5, EP₁, EP₂, EP₃, and EP₄ were 94 °C for 30 sec initial melt, 94 °C melt (30 s), 55 °C anneal and extend (60 s), for 30 cycles, with a final extension of 72 °C for 2 min. For K_{ir} 6.1, K_{ir} 6.2, SUR 1, and SUR 2B the conditions used were 94 °C for 30 sec initial melt, 94 °C melt (30 s), 50 °C anneal and extend (60 s), for 30 cycles, with a final extension of 72 °C for 2 min.

cAMP assay

ICCs were preincubated with 100 µM IBMX for 30 min at 37 °C to inhibit cAMP degradation and then incubated with PGE₂ (5 µM) for 10 min. The sample was extracted by homogenization in buffer containing 4 mM EDTA to prevent enzymatic cAMP degradation, followed by heating for 5 min in a boiling water bath to coagulate protein. After centrifugation at 3,000 rpm for 5 min, the cAMP in the supernatants was transferred into a new tube and stored at 4°C. The samples were assayed for cAMP using the [³H]- cAMP assay system (Amersham Pharmacia Biotech, Little Chalfont, UK).

Measurement of intracellular Ca²⁺ concentration

Changes intracellular Ca²⁺ concentration ([Ca²⁺]_i) were monitored by using fluo-3/AM, which was initially dissolved in dimethyl sulfoxide and stored at -20 °C. The cultured ICC on coverslip (25 mm) were rinsed twice with a bath solution [in mM: KCl 5, NaCl 135, CaCl₂ 2, glucose 10, MgCl₂ 1.2 and HEPES 10, adjusted to pH 7.4 with tris], incubated in the bath solution containing 5 µM fluo-3/AM with 5% CO₂-95% O₂ at 37 °C for 20 min, rinsed two more times with the bath solution, mounted on a perfusion chamber, and scanned every second with a confocal microscope (×200; fluoviews 300, Olympus). Fluorescence was excited at 488 nm, and emitted light was observed at 515 nm. During scanning of Ca²⁺ imaging, the temperature of the perfusion chamber containing the cultured ICC was kept at 30 °C. The variations of intracellular Ca²⁺ fluorescence emission intensity were expressed as F1/F0 that F0 means the intensity of first imaging.

Statistical analysis

Data were expressed as means ± 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

Effect of PGE₂ on pacemaker currents in ICC

Previous reports have described that the naturally occurring prostaglandins (PGs) comprise PGs D₂, E₂, F_{2α}, I₂ and TXA₂ [25]. In this study, because of their diverse action on the gastrointestinal tract, we checked the action of PGE₂ on pacemaker currents in cultured ICC. Under control conditions at a holding potential of -70 mV, the frequency, amplitude, and resting current levels were 13 ± 1.4 cycles/min, -360 ± 42 pA, and -27 ± 9 pA, respectively. When PGE₂ (1 µM) was applied to ICC, both the frequencies and the amplitudes of the pacemaker currents decreased, and the resting currents increased in the outward direction under voltage-clamp conditions (98 ± 11.4 pA) (Fig. 1A and B). In addition, the corresponding frequencies and amplitude under these conditions were

Fig. 1. Effects of prosta-glandins (PGs) E_2 on pacemaker currents and potentials in cultured ICC of the murine small intestine. (A) Under control conditions at a holding potential of -70 mV, PGE_2 ($1 \mu M$) inhibited the amplitude and the frequency of pacemaker currents and increased the resting currents in the outward direction in ICC. Also, glibenclamide ($10 \mu M$) blocked the actions of PGE_2 on pacemaker currents in ICC. (B), (C), and (D) summarize the inhibitory effects of PGE_2 on pacemaker currents. Bars represents means $\pm$ SE ($n = 9$). *Asterisks are significantly different from the control ($p < 0.05$). Dotted lines indicate zero current levels. (E) Pacemaker potentials from ICC exposed to PGE_2 ($1 \mu M$) in current clamp mode ($I=0$). PGE_2 caused membrane hyperpolarization and decreased amplitude of pacemaker potentials. Glibenclamide ($10 \mu M$) also blocked the effects of PGE_2 on pacemaker potentials in ICC.

2.1 ± 1.8 cycles/min and -20.9 ± 16 pA ($n=9$) (Fig. 1C and D), respectively. Also, in current clamp mode ($I=0$), we examined the effect of PGE_2 on membrane potentials and pacemaker potentials of ICC. PGE_2 produced membrane hyperpolarization and decreased the amplitude of the pacemaker potentials (Fig. 1E). These results suggested that PGE_2 modulates pacemaker currents in cultured ICC. Furthermore, we found that the PGE_2 -induced effects were returned to the base-line level by treating with glibenclamide ($10 \mu M$), the ATP-sensitive K^+ channels blocker (Fig. 1A and 1E).

Dose-dependency of the action of PGE_2 on pacemaker currents in ICC

In previous studies, we found that PGE_2 ($1 \mu M$) had an inhibitory effect on pacemaker currents in cultured ICC. In the present study, we tested that whether PGE_2 has a dose-dependent inhibitory effect on pacemaker currents in cultured ICC. Under a voltage clamp at a holding potential of -70 mV, ICC generated spontaneous

inward currents. The mean frequency of these pacemaker currents was 13 ± 1.4 cycles/min and their mean amplitude and mean resting current level were -360 ± 42 pA and -27 ± 9 pA, respectively ($n = 6$). The addition of 10 or 100 nM PGE_2 slightly reduced the amplitude and frequency of these pacemaker currents; 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. 2E, F and G), respectively. The presence of 10 or 100 nM PGE_2 slightly increased resting currents in the outward direction (Fig. 2A and B). In the presence of 1 or 10 μM PGE_2 under voltage-clamp conditions, pacemaker currents were largely inhibited and resting currents were increased in the outward direction (Fig. 2C and D). The inhibitory frequencies and amplitudes by PGE_2 were 2.1 ± 1.8 cycles/min and -20.9 ± 16 pA at 1 μM PGE_2 and 1.8 ± 1.4 cycles/min and -16 ± 19 pA at 10 μM PGE_2 , respectively. The resting current levels were 95 ± 5.9 pA

Fig. 2. The dose-dependent effects of PGE₂ on pacemaker currents in cultured ICC of the murine small intestine. (A), (B), (C), and (D) show pacemaker currents of ICC exposed to PGE₂ (0.01, 0.1, 1, or 10 μM) at a holding potential of -70 mV. PGE₂ inhibited spontaneous pacemaker currents in a dose-dependent manner in ICC and increased resting currents in the outward directions. (E), (F), and (G) summarize the inhibitory effects of PGE₂ on pacemaker currents in ICC. Bars represent means ± SE (n = 6-7/group). *Asterisks mean significantly different from the controls (p < 0.05), and dotted lines indicate zero current levels.

at 1 μM PGE₂ and 90 ± 7.2 pA at 10 μM PGE₂ (n = 7) (Fig. 2E, F and G). These results suggest that PGE₂ inhibits pacemaker currents in a dose-dependent manner in cultured ICC.

Localization of and the molecular expression of ATP-dependent K⁺ channels in ICC

Recently, we showed that pinacidil inhibits pacemaker currents, and activates outward currents, which are antagonized by glibenclamide, suggesting that ATP-dependent K⁺ channels exist in ICC, and that the activity of ATP-dependent K⁺ channels in ICC may be involved in the action of pacemaker currents [18]. In this

study, K_{ir} subunits and SURs were identified by RT-PCR method. To determine the presence of K_{ir} subunits and SURs, RT-PCR with c-kit positive single cell was performed using K_{ir} 6.1, K_{ir} 6.2, SUR1, and SUR2B gene-specific primers in ICC. In case of smooth muscle cell, the expression of K_{ir} 6.2 and SUR2B was predominant (Fig. 3A). Also, in ICC, RT-PCR detected transcripts for K_{ir} 6.2 and SUR2B (Fig. 3B), but the specific primers K_{ir} 6.1 and SUR1 did not produce cDNA fragments of the appropriate size in smooth muscle and ICC cell (Fig. 3A and B). This finding indicates that ATP-dependent K⁺ channels exist in cultured ICC and that they are composed of K_{ir} 6.2 and SUR 2B.

Fig. 3. Agarose gels of the RT-PCR products of the subunits of ATP-dependent K⁺ channels using single smooth muscle and ICC cell. This representative 1.2 % agarose gel was loaded with 5 ml of PCR product and stained with ethi-dium bromide. The markers shown in lane indicate bp. (A) K_{ir} 6.2 and SUR 2B primers produced the expected products in single smooth muscle cell (lanes 5 and 7) (K_{ir} 6.2: 340 bp, SUR 2B: 347 bp). However, K_{ir} 6.1 and SUR 1 primers failed to produce their respective product bands. (B) K_{ir} 6.2 and SUR 2B primers produced the expected products in single ICC (lanes 5 and 7) (K_{ir} 6.2: 340 bp, SUR 2B: 347 bp). However, K_{ir} 6.1 and SUR 1 primers failed to produce their respective product bands.

Characterization of the EP receptor subtypes, involved in the effects of PGE₂ on pacemaker currents in ICC

Four subtypes of EP receptor have been identified to date, and have been arbitrarily named EP₁, EP₂, EP₃, and EP₄. In the present study, we attempted to discern which of these EP receptor subtypes mediate the inhibitory actions of PGE₂ on pacemaker currents in cultured ICC. First, we examined the effects of butaprost, a specific agonist for the EP₂ receptor subtype, on pacemaker currents in cultured ICC. The addition of butaprost (1 μM) caused a reduction in spontaneous inward currents frequencies and amplitudes in cultured ICC (Fig. 4A) and increased resting currents in the outward direction (n = 5) (control vs. butaprost; resting currents, -56 ± 5 pA vs. 86 ± 5.4 pA; amplitudes -370 ± 39 pA vs. -25 ± 12 pA; and frequencies, 15 ± 1.9 cycles/min vs. 3 ± 2.7 cycles/min) (bar graph not shown). These results are similar to those of PGE₂ treatments (as shown in figure 1) and also glibenclamide pre- or co-treatment (1 μM, an ATP-dependent K⁺ channel blocker) blocked the effects of butaprost on pacemaker currents (Fig. 4A and B). Sulprostone (an EP₃ and EP₁ receptor agonist; 1 μM) had no effects on the frequency or amplitude of pacemaker currents in ICC, and had no effects on resting pacemaker currents in cultured ICC (data not shown). In addition, the pretreatment of ICC with either SC19220, an EP₁ receptor antagonist, (1 μM) or co-treatment with SC-19220 (1 μM) and sulprostone (1 μM) did not have any effects on pacemaker currents (Fig. 4C). Also, PCR

assays with c-kit positive single cell using EP₂ receptors primers yielded a product of the appropriate size (245 bp). We found that the EP₂ PCR product was produced from c-kit positive single cell, but that EP₁, EP₃, and EP₄ were never amplified (Fig. 4D). These results suggest that PGE₂ may affects ATP-dependent K⁺ channels in ICC by stimulating EP₂ subtype receptors.

PGE₂-induced pacemaker currents inhibition is not mediated via cAMP pathway

To investigate the involvement of cAMP on the effects of PGE₂ in ICCs, we used SQ-22536, an inhibitor of adenylate cyclase, cell-permeable 8-bromo-cAMP, and cAMP assays. The preincubation of ICCs with SQ-22536 (100 μM) for 10 min had no effects on the control states of the pacemaker currents, and co-treatment with SQ-22536 (100 μM) and PGE₂ (1 μM) inhibited the pacemaker currents (n = 5) (Fig. 5A), thus indicating that SQ-22536 had no influence on PGE₂-induced pacemaker currents inhibition. In addition, the cell-permeable 8-bromo-Camp (100 μM) had no effect on the generation of pacemaker currents (n = 4) (Fig. 5B). Moreover, to evaluate whether a change in cAMP content is involved in the effect of PGE₂ on ICCs, intracellular cAMP contents were measured under basal and PGE₂-stimulated conditions. 5 μM of PGE₂ did not stimulate cAMP production (control: 12.5 ± 1.5 vs. PGE₂: 13.1 ± 2.9 pmol/mg⁻¹ protein) (Fig. 5C). These results indicate that cyclic AMP does not mediate the actions of pacemaker currents induced by PGE₂.

Fig. 4. Effects of EP₂ (butaprost) and EP₃ (sulprostone) receptor agonists on spontaneous inward currents in cultured ICC and agarose gels of RT-PCR products of EP receptor subtypes in single ICC cell. (A) Butaprost (1 μ M) reduced the frequency and amplitude of spontaneous inward currents and increased resting currents in the outward directions. (A) and (B) The effects of butaprost (1 μ M) on pacemaker currents disappeared by co-treating or pre-treating with glibenclamide (10 μ M). (C) Sulprostone (1 μ M) with EP₁ antagonists, SC 19220 (10 μ M) pretreating had no effects on pacemaker currents on pacemaker currents. Dotted lines indicate zero current levels. (D) This representative 1.2 % agarose gel was loaded with 5 μ l of PCR product and stained with ethidium bromide. The markers shown in lane indicate bp. Only EP₂ primers produced the expected products in single ICC cell (lanes 5) (EP₂: 225 bp). However, EP₁, EP₃, and EP₄ primers failed to produce their respective product bands. GBC: glibenclamide.

Involvement of $[Ca^{2+}]_i$ on PGE_2 -induced action in ICC

Because many reports suggested $[Ca^{2+}]_i$ oscillations in ICC are considered to be the primary mechanism for the pacemaker activity in gastrointestinal activity, we examined the effect of PGE_2 on $[Ca^{2+}]_i$ oscillations in ICC. In this study, we measured spontaneous $[Ca^{2+}]_i$ oscillations of ICC which are connected with cell clusters. Spontaneous $[Ca^{2+}]_i$ oscillations observed in many ICC (Fig. 6A) which was loaded with fluo3-AM. And in the presence of 1 μ M PGE_2 , $[Ca^{2+}]_i$ oscillations in ICC rapidly was declined (Fig. 6C). Also, spontaneous $[Ca^{2+}]_i$ oscillations inhibited by PGE_2 in ICC was recovered by co-treatment of 10 μ M glibenclamide (Fig. 6E). The data of time series are summarized in Fig. 6B, D, and F). These results suggest that the action of PGE_2 on ICC may involve the regulation of spontaneous $[Ca^{2+}]_i$ oscillations.

Discussion

Prostaglandins (PGs) act as local regulatory agents, and control smooth muscle contractile activity. Moreover, PGE_2 has been shown to contract intestinal longitudinal

smooth muscle and relax circular smooth muscle [26, 27], implying that PGE_2 may regulate gastrointestinal motility. Furthermore, since ICC generate electrical slow waves that are the basic determinants of gastrointestinal motility, PGE_2 may have effects on ICC slow waves and control gastrointestinal motility. In the present study, we investigated pacemaker currents inhibition by PGE_2 by modulating ATP-dependent K^+ channels composed of K_{ir} 6.2 and SUR 2B in ICC and characterized the EP₂ receptor subtypes involved in the inhibition of pacemaker currents by PGE_2 . We also showed the action of PGE_2 on pacemaker currents in ICC was not mediate via cAMP pathway and involved the regulation of spontaneous $[Ca^{2+}]_i$ oscillations generated by ICC.

Fatty acid cyclooxygenase in the gastrointestinal tract converts eicosatetraenoic acid (arachidonic acid) primarily to prostacyclin (prostaglandin I₂), and, to a lesser extent, to PGE_2 , $PGF_2\alpha$, and thromboxane A₂ [28-31]. Previous studies on gastrointestinal tract motility have shown that PGE_2 generally contracts the longitudinal smooth muscle layer of the small intestine and relaxes the circular muscle layer [26, 27]. In the present study, we found that PGE_2 promotes outward pacemaker currents in ICC (Fig. 1A). Moreover, recent reports have shown that deoxycholic

Fig. 5. Effects of adenylate cyclase on pacemaker currents, and the effects of PGE₂ on cAMP production in cultured ICC. (A) Pretreatment with SQ-22536 (100 μ M) did not affect the inhibitory effects of PGE₂ (1 μ M) on spontaneous inward currents. Dotted lines indicate zero current levels. (B) Effect of 8-bromo-cAMP (100 μ M) on pacemaker currents. 8-bromo-cAMP had no effect on pacemaker currents. (C) Preincubation of ICCs with PGE₂ (5 μ M) did not significantly stimulated cAMP production. Bars represents means $\pm$ SE of three independent experiments conducted in triplicate.

acid inhibits ICC pacemaker currents by activating ATP-dependent K⁺ channels by inducing PGE₂ production [18]. Specifically, this report suggests that PGE₂ affects pacemaker currents by modulating ATP-dependent K⁺ channels in ICC. Taken together, PGE₂ does modulate gastrointestinal tract motility, and this action depends on species and PGE₂ concentration [5-7].

In terms of its concentration, PGE₂ has been shown to have dual effects on the colonic motility of rabbit *in vivo* and *in vitro*; i.e., a suppressive effects at low concentration, and an activating effect at high concentration [32] and similar effects on stomach activity in a guinea-pig model [33]. Those results mean that PGE₂ have various actions depending on concentration, the organ, and the species. In ICC from murine small intestine, we found that PGE₂ have only a dose-dependent inhibitory effects on pacemaker currents (Fig. 2) and, a slight or no inhibitory effect on pacemaker currents at 1 nM or 100 pM (data not shown).

Many studies have found that ATP-dependent K⁺ channels play important roles in regulating resting membrane potential and membrane excitability in a variety of tissues. In addition, Jun et al [18] in an electrophysiological study reported on the role of ATP-dependent K⁺ channels in ICC. ATP-dependent K⁺ channels are heteromultimers comprised of inwardly rectifying K⁺ channel subunits (K_{ir} 6.x) and sulfonylurea receptors

(SURs). Various combinations of these two subunits convey the heterogeneity of channel properties observed in native cells, such as K_{ir} 6.2-SUR 1 in pancreatic β -cells, K_{ir} 6.2-SUR 2A in cardiac and skeletal muscles, and K_{ir} 6.1-SUR 2B or K_{ir} 6.2-SUR 2B in smooth muscle [34]. In this study, we identified ATP-dependent K⁺ channels subunits using RT-PCR with c-kit positive single cell. By using single cell RT-PCR assay, we observed K_{ir} 6.2 and SUR 2B mRNA transcripts (Fig. 3B) in ICC. Also, in smooth muscle cell, we found K_{ir} 6.2 and SUR 2B mRNA transcripts (Fig. 3A). Interestingly, there are many reports that give rise to much controversy. Especially, Koh et al (1998) [35] suggested the presence of K_{ir} 6.2 and SUR 2B subunits in colonic smooth muscle cells same as our results but Nakayama et al (2005) [36] showed that RT-PCR examinations revealed predominant expression of K_{ir} 6.1 and SUB 2B in smooth muscle, with predominant expression of K_{ir} 6.1 and SUR 1 in ICC. While we could not make clear the difference our and previous suggestions, we thought that our results that was verified using single cell are more exact contrary to previous reports. Therefore, our results indicate that the effects of PGE₂ on pacemaker currents in ICC occur via ATP-dependent K⁺ channels comprised of K_{ir} 6.2-SUR 2B.

The recent cloning and expression of PG receptors has confirmed not only the existence of at least four of

Fig. 6. Intracellular Ca^{2+} waves in cultured ICC. (A) Sequential fluorescence intensity images of fluo-3-loaded cultured ICC in normal condition. The interval of representative frame was 1 min and the exposure time of each frame was 200 ms. (B) Fluorescence intensity change plotted in (A) red marker. (C) Sequential fluorescence intensity images of fluo-3-loaded cultured ICC in presence of PGE_2 ($1 \mu\text{M}$). (D) Fluorescence intensity change plotted in (C) red marker. (E) Sequential fluorescence intensity images of fluo-3-loaded cultured ICC in co-treatment of PGE_2 ($1 \mu\text{M}$) and glibenclamide ($10 \mu\text{M}$). (F) Fluorescence intensity change plotted in (E) red marker.

five classes of prostaglandin receptor (IP for PGI_2 binding, FP for $\text{PGF}_2\alpha$ 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 . Several agonists and antagonists are known for the EP receptor. To date, butaprost appears to be the most selective EP_2 receptor subtype agonist [37], and sulprostone is known to agonist the EP_1 and EP_3 receptors [38]. Moreover, ONO-AE-248 antagonizes the EP_4 receptor, and SC19920 the EP_1 receptor [39], but as yet no antagonists of the EP_2 and EP_3 receptors have been identified. Interestingly, it was reported that EP_2 , EP_3 , and EP_4 agonists affect pacemaker current

frequencies in the murine gastric antrum [40]. However, in the murine small intestine, only butaprost was found to have an inhibitory effect on cultured ICC pacemaker currents, and this effect was similar to that of PGE_2 (Fig. 4A). Without sulprostone, the pretreatment of ICC with SC19920 or co-treatment with SC19920 and sulprostone (to block the EP_1 receptor) had no effects on spontaneous inward currents (Fig. 4C). Furthermore, RT-PCR assays using single cell only amplified the EP_2 primer (Fig. 4D). As described above, the action of PGE_2 varies greatly, and depends on its concentration, the organ, the species, and even the muscle layer studied [5-7]. Therefore, we suggest that only the EP_2 receptor subtypes affect

pacemaker currents in murine small intestine, and that this differs from the situation in the gastric antrum. Also, in as shown in fig. 4A and B, we found that pre- or co-treatment with glibenclamide (ATP-dependent K^+ channel blocker) blocked the effects of butaprost on pacemaker currents in ICC. This result suggests that the stimulation of EP_2 receptors in ICC may regulate the activities ATP-dependent K^+ channels.

Almost all of the studies of PG and second messengers until the late 1980s were concerned with cyclic nucleotides, particularly cAMP. Butcher and colleagues were the first to demonstrate an association between PGs and cAMP [41, 42], and although their observation made little initial impact, it became increasingly accepted that E-series PGs at least were capable of stimulating adenyl cyclase to cause increases in intracellular cAMP [43, 44]. Several reports suggested the participation of cAMP on PGE_2 actions, especially the EP_2 receptor. The results by Hardcastle et al. (1982) provide direct evidence for positive coupling of an EP receptor to adenylate cyclase in their demonstration of an association between EP_2 receptors and cAMP generation in enterocytes [45] and similarly it was found an association between EP_2 receptor stimulation and cAMP generation in corneal endothelial cells [46]. Furthermore, in cells expressing the recombinant murine EP_2 receptor, PGE_2 increased the intracellular cAMP level without any change in inositol phosphate content [47]. These several reports predict that, on pacemaker currents in ICC, PGE_2 may have the actions of cyclic nucleotides signaling pathway. Namely, in ICC, the generation of pacemaker currents and the regulation of ATP-sensitive K^+ channels on this may involve the cAMP signaling. However, in a recent study and figure 5B, the treatment 8-bromo-cAMP (a cell-permeable cAMP analog) in ICC had no effects on pacemaker currents [48]. Also, pretreatment with SQ-22536, an adenylate cyclase inhibitor, does not influence PGE_2 actions on pacemaker currents, and in a cAMP assay, PGE_2 did not stimulate the production of cAMP. Taken together, PGE_2 appears to function in diverse cells and tissues by modulating a cAMP-dependent pathway. However, in ICC, PGE_2 has an inhibitory effect on pacemaker currents that is independent of the cAMP pathway. Further studies on the actions of PGE_2 in ICC are needed, especially on

second messenger.

Recent studies have suggested that pacemaker activity depends on a link between Ca^{2+} release from cellular stores, oxidative metabolism, and the pacemaker conductance in the plasma membrane [49]. Especially, we noted the inositol 1,4,5-triphosphate receptor plays a role in generating spontaneous electrical activity in gastrointestinal pacemaker cells [15] and previous suggestion that periodic Ca^{2+} release from intracellular Ca^{2+} stores produces $[Ca^{2+}]_i$ oscillations in ICC, using cell cluster preparations isolated from mouse ileum [50] and these actions seen in ICC are considered to be the primary pacemaker activity in the gut. But, we thought that small cell clusters that show stable spontaneous rhythmicity in terms of mechanical, electrical and intracellular Ca^{2+} activities contain c-kit immunopositive ICC, smooth muscle cells and enteric neurons. So, in this study we examined $[Ca^{2+}]_i$ oscillations of ICC that are branched with cluster. In fig. 6, we could examine the spontaneous $[Ca^{2+}]_i$ oscillations in ICC and find that PGE_2 inhibited the $[Ca^{2+}]_i$ oscillations. The actions of PGE_2 on $[Ca^{2+}]_i$ oscillations in ICC are in keeping with the suggestions that $[Ca^{2+}]_i$ oscillations are important actions of pacemaker activity. Taken together, we conjecture that the opening of ATP-sensitive K^+ channels by PGE_2 hyperpolarize membrane potentials of ICC and this action evokes inhibition of periodic Ca^{2+} release from intracellular Ca^{2+} stores that are considered to be the primary pacemaker activity in ICC.

In conclusion, the present results indicate that PGE_2 directly alters pacemaker currents by modulating ATP-dependent K^+ channels comprised of K_{ir} 6.2- and SUR 2B in ICC. Moreover, this affects of PGE_2 on pacemaker currents is not mediated via cyclic nucleotides-dependent pathway and involved by the activation of EP_2 receptors and mobilization of $[Ca^{2+}]_i$.

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