Original Paper

Pharmacological Differences of Endothelin Receptors-mediated Modulation in Cultured Interstitial Cells of Cajal from the Murine Small and Large Intestine

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Key Words

Interstitial Cells of Cajal • Pacemaker potentials • Endothelin • ET_A receptor • ET_B receptor • Intestinal motility

Abstract

Interstitial cells of Cajal (ICCs) are pacemaker cells that activate the periodic spontaneous depolarization (pacemaker potentials) responsible for the production of slow waves in gastrointestinal smooth muscle. Under current clamping, ICCs had a mean resting membrane potential of -58 ± 3 mV and externally applied ET produced membrane depolarization in a dose-dependent manner. These effects were reduced by intracellular GDP beta S. A comparison of the concentration-dependent membrane depolarizations on pacemaker potentials to ET-1, ET-2 and ET-3 showed a rank order of potency $ET-1 \geq ET-2 \geq ET-3$ in cultured murine small intestinal ICCs. The pretreatment with Ca^{2+} -free solution and thapsigargin, a Ca^{2+} -ATPase inhibitor in endoplasmic reticulum, abolished the generation of pacemaker potentials and suppressed the ET-1 induced membrane depolarizations. Chelerythrine and calphostin C, protein kinase C inhibitors or naproxen, an inhibitor of cyclooxygenase, did not block the ET-1 induced effects on pacemaker potentials. Pretreatment with BQ-123 (ET_A receptor antagonist) or BQ-788 (ET_B receptor antagonist) blocked the ET-1 induced effects on pacemaker potentials in cultured murine small intestinal ICCs. However, pretreatment with BQ-788 selectively did not block the ET-1 induced effects on pacemaker potentials in cultured murine large intestinal ICCs. Also, only externally applied selective ET_B receptor agonist, IRL 1620 did not show any influence on pacemaker potentials in cultured murine large intestine ICCs. RT-PCR results indicated the presence of the ET_A and ET_B receptor in ICCs. These results suggested that ET-1 modulates pacemaker potentials through ET_A and ET_B receptor activation in murine small intestinal ICCs and ET_A receptor activation in murine large intestinal ICCs by external Ca^{2+} influx and internal Ca^{2+} release via protein kinase C or cyclooxygenase-independent mechanism. Therefore, the ICCs are targets for ET and their interaction can affect intestinal motility.

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Introduction

The gastrointestinal (GI) tract shows spontaneous mechanical contractions, mediated by the periodic generation of electrical pacemaker potentials, which are basic determinant of GI smooth muscle activity [1]. Recent studies have shown that the interstitial cells of Cajal (ICCs) act as the pacemakers and conductors of the electrical slow waves in GI smooth muscles [2-6]. Moreover there is evidence that endogenous agents such as neurotransmitters, hormones and paracrine substances modulate GI tract motility by influencing the ICCs [7-11].

Endothelins (ETs) are a family of peptides with 21 amino acid residues. ET-1 was originally identified in 1988 as a potent vasoconstrictor produced by vascular endothelial cells [12, 13]. The ET system comprises three potent, vasoactive autacoids named ET-1, ET-2 and ET-3, several isoforms of ET converting peptidases, and two G-protein coupled transmembrane receptors: ET_A and ET_B [14-16]. Of the three ET isoforms (1, 2, and 3), ET-1 has been studied most extensively. ET-1 is expressed in endothelial cells, cardiomyocytes, smooth muscle cells, epithelial cells of lung, renal tubules, intestine, urinary tract, uterus, and in neutrophils, monocytes, mast and Sertoli cells [17]. ET-1 regulates function of the blood vessels and heart whereas in the kidney it modulates vascular and mesangial tone, water and sodium turnover and cell proliferation [18-20]. In the literature there are strong data indicating on the involvement of ETs in physiology and pathophysiology of GI tract. ET-1 is expressed in GI tissues including vascular endothelium, submucosa stroma and circularis muscularis layers of the gut [21]. The effects of ET-1 on smooth muscle are mediated through at least two distinct subtypes of receptors, namely the endothelin type A (ET_A) receptors and the endothelin type B (ET_B) receptors [22]. ET-1 acts through the G-protein-IP₃ pathway, which increases intracellular free Ca²⁺ by triggering influx from outside of the cells and the release of Ca²⁺ from intracellular stores [17]. The mounting interest in ET research, coupled with the discovery of potent agonists and antagonists, may lead to a better understanding of the pathogenic mechanisms in the GI tract and the development of novel therapies for GI disorders. Moreover, the discovery of potent ET antagonists and the recent demonstration of their effectiveness in animal models of pathology such as Hirschsprung's disease [23], has provided preliminary evidence that endogenous ETs may be of importance in the pathogenesis of human diseases [17, 24, 25]. The following studies have reported the diverse effects of ET-1 on vascular and non-vascular smooth muscle. In several regions of the GI tract, ET-1 elicits biphasic effects on smooth muscle, with an initial relaxation followed by a sustained contraction [26-29]. ET-1 also has dual effects on slow waves in the rat gastric antrum, through different types of receptors. ET-1 hyperpolarizes the membrane via ET_A receptors, and depolarizes the membrane and increases the frequency of slow waves via ET_B receptors [30]. As these experiments were performed in intact tissues, it was difficult to differentiate between the effects on ICCs and those on smooth muscle. Therefore, in this study, we investigated the effects of ET on the electrical properties of cultured ICCs, in order to elucidate the mechanisms of the effects of ET on intestinal motility.

Materials and Methods

Materials

ET-1, ET-2, ET-3, BQ-123, BQ-788, and IRL 1620 were purchased from TOCRIS (Bristol, UK). Chelerythrine, calphostin C and naproxen were purchased 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 cell cultures

All experiments were carried out according to the guiding principles for the care and use of animals approved by the ethics committee in Pusan National 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 (7-12 days old) of either sex were anaesthetized with ether

and sacrificed by cervical dislocation. The small intestines from 1 cm below the pyloric ring to the caecum were removed and opened along the mesenteric border. 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 tissue stripes of intestine muscle (consisting of 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 25 min. Then, the cells were dispersed with an enzyme solution containing collagenase (Worthington Biochemical Co, Lakewood, NJ, U.S.A.) 1.5 mg/ml, bovine serum albumin (Sigma Chemical Co., St Louis, MO, U.S.A.) 3 mg/ml, trypsin inhibitor (Sigma) 1.5 mg/ml and ATP 0.3 mg/ml. Cells were plated onto sterile glass coverslips coated with murine collagen (2.5 $\mu\text{g}/\text{ml}$, Falcon/BD) in a 35 mm culture dish. The cells were then cultured at 37°C in a 95% O_2 -5% CO_2 incubator in a smooth muscle growth medium (SMGM, Clonetics Corp., San Diego, CA, U.S.A.) supplemented with 2% antibiotics/antimycotics (Gibco, Grand Island, NY, U.S.A.) and murine stem cell factor (SCF, 5 ng/ml, Sigma). ICCs were identified immunologically with anti-c-kit antibody (phycoerythrin (PE)-conjugated rat anti-mouse c-kit monoclonal antibody; eBioscience, San Diego, CA, USA) at a dilution of 1:50 for 20 min [31]. The morphologies of ICC were distinct from other cell types in the culture, so it was possible to identify the cells with a phase contrast microscopy once the cells had been confirmed with anti-c-kit antibody.

Patch-clamp experiments

The physiological salt solution used to bathe cells (Na^+ -Tyrode) contained (mM): KCl 5, NaCl 135, CaCl_2 2, glucose 10, MgCl_2 1.2 and HEPES 10, adjusted to pH 7.4 with NaOH. The pipette solution contained (mM): KCl 140, MgCl_2 5, K_2ATP 2.7, NaGTP 0.1, creatine phosphate disodium 2.5, HEPES 5 and EGTA 0.1, adjusted to pH 7.2 with KOH. The whole-cell configuration of the patch-clamp technique was used to record membrane potentials (current clamp) of cultured ICC. Axopatch I-D (Axon Instruments, Foster, CA, USA.) amplified membrane currents and potentials. The command pulse was applied using an IBM-compatible personal computer and pClamp software (version 6.1; Axon Instruments). The data were filtered at 5kHz and displayed on an oscilloscope, a computer monitor and with a pen recorder (Gould 2200, Gould, Valley view, OH, USA.). Results were analyzed using pClamp and Origin (version) software. All experiments were performed at 30 °C.

RT-PCR with c-Kit positive cell and smooth muscle 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 extracted by using TRIzol reagent (Invitrogen), and reverse transcription of total RNA was performed by using random hexamer primers and Superscript II-RT (Life Technologies Inc), according to the manufacturer's instructions. Primers used as follows : ET_A (Accession number NM_010332), forward 5' - CCC AAA ACC TCC CAA GTC TCT C - 3', reverse 5' - TGG AAA TGA CAT GCG CGG TAT - 3', (with a product size of 159 bp)-; ET_B (Accession number NM_007904), forward 5' - CCC TTC ACC TCA GCC GGA TCC T - 3', reverse 5' - GGC ACC AGC AGC ACA AAC ATG A - 3', (with a product size of 203 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 179 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 195 bp), and PGP9.5 (Accession number AF172334), forward 5' - AAA GCA AAT TGA GGA ACT GA - 3', reverse 5' - GTT CTT CTC GAA ACA CTT GG - 3', (with a product size of 216 bp). Primers were performed for 40 cycles under the following conditions: denaturing at 94°C for 2 minutes, annealing at 50°C for 1 minute, and polymerization at 72°C for 1 minute. The identification of the PCR products was confirmed by DNA sequencing.

Immunohistochemistry

Cultured ICCs from small intestine of Balb/C were used for immunohistochemistry. Cultured ICCs were fixed in cold acetone (4°C) for 5 min. After fixation, they were washed in phosphate-buffered saline (PBS; 0.01 M, pH 7.4) and immersed in 0.3% Triton X-100 in PBS. After blocking with 1% bovine serum albumin (BSA; Sigma) in 0.01 M PBS for 1 hour at room temperature, they were incubated with a goat antibody raised against c-Kit (R&D systems, Inc) at 4 $\mu\text{g}/\text{ml}$, or rabbit antibody against VPAC1 and VPAC2 (Santa cruz) for 24 hours (4°C). After rinse in PBS at 4°C, they were labeled with the donkey anti-goat Alexa Fluor 568 (Molecular

Fig. 1. Effects of endothelin (ET)-1 on pacemaker potentials in cultured ICC of the murine small intestine. (A-C) Pacemaker potentials from ICC exposed to ET-1 (1–30 nM) in the current-clamp mode ($I=0$). ET-1 caused a concentration-dependent membrane depolarization and decreased the frequency and amplitude of pacemaker potentials. Responses to ET-1 are summarized in (D). Bars represent mean values $\pm$ S.E. * $P<0.05$, ** $P<0.01$: significantly different from the untreated control. The dotted lines indicate zero current levels.

Probe, 1 $\mu\text{g/ml}$, red color) or donkey anti-rabbit Alexa Fluoro 488 (Molecular Probe, 1 $\mu\text{g/ml}$, green color) for 1 h at room temperature. For double immunostaining, specimens were incubated with a mixture of antibody raised against VPAC1 or VPAC2 and antibody raised against c-kit for 24 h at 4°C. After a thorough wash with PBS, the mixture of labeled secondary antibodies was incubated for 1 h at room temperature. Tissues were examined with laser scanning confocal microscope (FV 300, Olympus, Japan) with an excitation wavelength appropriate for Alexa Fluoro (568 nm or 488 nm). Final images were constructed with Flow-View software (Olympus, Japan).

Statistics

All data are expressed as mean $\pm$ S.E. Student's *t*-test for unpaired data was used to compare the control and the experimental groups. A *P* value of less than 0.05 was considered to indicate statistically significant differences. The *n* values reported in the text refer to the number of cells used in experiments.

Results

Effects of endothelin (ET)-1 on pacemaker potentials in cultured ICCs of the murine small intestine

Under a current clamp mode ($I = 0$), ICC generated pacemaker potentials. The resting membrane potential was -58 ± 3 mV and amplitude was 22 ± 3 mV. ET-1 (1–30 nM) produced membrane depolarization and decreased the frequency and amplitude of pacemaker potentials in a concentration-dependent manner (Fig. 1A–C). The addition of ET-1 1 nM, there are no any responses (Fig. 1A). But, in the presence of ET-1 10 nM or 30 nM, membrane depolarizations were 23.6 ± 2 mV and 53.1 ± 3 mV, respectively (Fig. 1B and 1C, $n = 7$). The summarized values and bar graph of ET-1 effects on pacemaker potentials are shown in Fig. 1D. These results suggest that ET-1 modulated pacemaker activity in a dose-dependent manner in ICCs.

Fig. 2. A comparison of the concentration-dependent membrane depolarization to ET-1, ET-2 and ET-3. (A, B) Pacemaker potentials from ICC exposed to ET-2 (10 nM) and ET-3 (10 nM) in the current-clamp mode ($I=0$). ET-2 and ET-3 caused a concentration-dependent membrane depolarization and decreased the frequency and amplitude of pacemaker potentials. (C) Responses to ET are summarized. Each point represents the means with the S.E. shown by vertical bars. **Asterisks are significantly different from the control ($p < 0.01$). Dotted lines indicate zero current levels.

Fig. 3. Effects of $\text{GDP}_{\beta}\text{S}$ on the response to ET-1. (A) Pacemaker potentials of ICCs exposed to ET-1 (10 nM) in the presence of $\text{GDP}_{\beta}\text{S}$ (1 mM) in the pipette. $\text{GDP}_{\beta}\text{S}$ partially blocked the ET-1 mediated membrane depolarization of pacemaker potentials. The effect of ET-1 in the presence of $\text{GDP}_{\beta}\text{S}$ but not in the pipette is summarized in (B) and (C). Bars represent mean values $\pm$ S.E.

A comparison of the concentration-dependent membrane depolarization to ET-1, ET-2 and ET-3

We compared the effects of three different ET types on pacemaker potentials in cultured ICCs. ET-2 and ET-3 (1–30 nM) also produced membrane depolarization and decreased the frequency and amplitude of pacemaker potentials in a concentration-dependent manner (Fig. 2A and 2B, $n = 8$). In the presence of ET-1, ET-2 and ET-3, membrane depolarizations were 23.5 ± 4 mV, 20.1 ± 4 mV and 19.1 ± 3 at 10 nM; 53.2 ± 5 mV, 48.1 ± 5 mV and 45.2 ± 3 at 30 nM; 55.1 ± 5 mV, 51.2 ± 4 mV and 50.2 ± 4 at 100 nM; 56.2 ± 5 mV, 53.1 ± 4 mV and 52.0 ± 5 at 300 nM, respectively. The summarized values of ET-1, ET-2 and ET-3 effects on pacemaker potentials are shown in Fig. 2C.

Involvement of G-proteins in ET-1 induced membrane depolarization of pacemaker potentials

The effects of $\text{GDP}_{\beta}\text{S}$, a nonhydrolysable guanosine 5'-di-phosphate analogue, which permanently inactivates GTP-binding proteins [32], were examined to determine whether the G-protein is involved in the effects of ET-1 in ICCs. The effect of ET-1 was reduced when $\text{GDP}_{\beta}\text{S}$ (1 mM) was in the pipette (Fig. 3A). Under control conditions, the addition of ET-1 (10 nM) caused the membrane depolarization and decreased the frequency on pacemaker potentials (membrane depolarization: 27.1 ± 4 mV; frequency: 1.1 ± 1 cycles min^{-1}). In the

Fig. 4. Effects of an external Ca^{2+} -free solution and thapsigargin upon ET-1 induced responses on pacemaker potentials in cultured ICCs of the murine small intestine. (A) The external Ca^{2+} -free solution abolished the generation of pacemaker potentials. Under this condition, ET-1 induced depolarizations were suppressed. (C) Thapsigargin (5 μM) abolished the generation of pacemaker potentials. However, thapsigargin blocked the ET-1 induced depolarization. Responses to ET-1 in the external Ca^{2+} -free solution and in the presence of thapsigargin are summarized in (B) and (D). Bars represent mean values $\pm$ S.E. **Asterisks are significantly different from the control ($p < 0.01$). Dotted lines indicate zero current levels.

presence of GDP_{β} S in the pipette, the effects of ET-1 on these variables were attenuated; the membrane depolarization and frequency were 10.2 ± 2 mV and 15.1 ± 2 cycles min^{-1} , respectively ($n = 5$) (Fig. 3B and 3C). These values were significantly different from those obtained with ET-1 alone. This indicates that G-proteins have an essential role in ET-1 induced effects on pacemaker potentials in ICCs.

Effects of external Ca^{2+} -free solution and Ca^{2+} -ATPase inhibitor of endoplasmic reticulum in ET-1 induced responses in cultured ICCs

To investigate the role of external Ca^{2+} or internal Ca^{2+} , ET-1 was tested under external Ca^{2+} -free conditions and in the presence of thapsigargin, a Ca^{2+} -ATPase inhibitor of endoplasmic reticulum. Pacemaker potentials were completely abolished by external Ca^{2+} -free solution. In this condition, ET-1 induced membrane depolarizations were suppressed ($n = 5$; Fig. 4A). In the external Ca^{2+} -free solution, the membrane depolarizations produced by ET-1 were 6.7 ± 2 mV. These values were significantly different when compared with ET-1 in normal Ca^{2+} solution (Fig. 4B). In addition, ET-1 induced membrane depolarizations were suppressed with pretreatment of thapsigargin ($n = 5$; Fig. 4C). In the presence of thapsigargin (5 μM), the membrane depolarizations produced by ET-1 were 7.3 ± 1 mV. These values were significantly different when compared to ET-1 in the absence of thapsigargin (Fig. 4D).

Effects of protein kinase C inhibitor or cyclooxygenase inhibitor in ET-1-induced responses in cultured ICCs

We tested the effects of chelerythrine and calphostin C or naproxen to investigate whether ET-1 induced responses to pacemaker potentials are mediated by the activation of protein kinase C or the production of prostaglandins. Chelerythrine (1 μM), calphostin C (1 μM) and naproxen (10 μM) did not have an effect on pacemaker potentials ($n = 5$; Fig. 5A-C). Pretreatment with chelerythrine, calphostin C and naproxen did not block the ET-1 induced membrane depolarizations of pacemaker potentials (Fig. 5D).

Fig. 5. Effects of chelerythrine, calphostin C (protein kinase C inhibitors) or naproxen (a cyclooxygenase inhibitor) upon ET-1 induced depolarizations on pacemaker potentials in cultured ICC of the murine small intestine. (A, B) Pacemaker potentials of ICC exposed to ET-1 (10 nM) in the presence of chelerythrine (1 μ M) or calphostin C (1 μ M). In this condition, ET-1 caused membrane depolarization and decreased the frequency and amplitude of pacemaker potentials. (C) Pacemaker potentials of ICC exposed to ET-1 (10 nM) in the presence of naproxen (10 μ M). Naproxen did not block the effects of ET-1 on pacemaker potentials. Responses to ET-1 in the presence of chelerythrine, calphostin C or naproxen are summarized in (D). Bars represent mean values $\pm$ S.E. The dotted lines indicate the zero current levels.

Identification of receptor subtypes of ET-1 in cultured ICCs

To identify the receptor subtypes of ET, ET receptor antagonists and agonists were used. ET_A receptor antagonist, BQ-123 (1 μ M) or ET_B receptor antagonist, BQ-788 (1 μ M) itself had no effect on pacemaker potentials. In the presence of BQ-123, ET-1 induced responses on pacemaker potentials in cultured murine small intestinal ICCs were completely blocked ($n = 5$; Fig. 6A). Also, BQ-788 almost completely blocked the ET-1 induced responses to pacemaker potentials ($n = 5$; Fig. 6B). Under controlled conditions, the membrane depolarizations on pacemaker potentials in cultured murine small intestinal ICCs produced by ET-1 were 23.6 ± 3 mV (Fig. 1B). In the presence of BQ-123, the membrane depolarizations on pacemaker potentials produced by ET-1 were 3.1 ± 2 mV. Also in the presence of BQ-788, the membrane depolarizations on pacemaker potentials produced by ET-1 were 3.3 ± 2 mV. The values of ET_A receptor antagonist, BQ-123 or ET_B receptor antagonist, BQ-788 were significantly different from those obtained in controlled conditions (Fig. 6F). We also investigated which receptor subtypes of ET were involved on pacemaker potentials in cultured murine large intestinal ICCs. BQ-123 (1 μ M) or BQ-788 (1 μ M) itself had no effect on pacemaker potentials, too. In the presence of BQ-123, ET-1 induced responses on pacemaker potentials in cultured murine large intestinal ICCs were completely blocked ($n = 5$; Fig. 6C). However, surprisingly, in the presence of ET_B receptor antagonist, BQ-788, ET-1 induced membrane depolarizations on pacemaker potentials were still present ($n = 5$; Fig. 6D). Under controlled conditions, the membrane depolarizations on pacemaker potentials in cultured murine large intestinal ICCs produced by ET-1 were 24.3 ± 3 mV. In the presence of BQ-123, the membrane depolarizations on pacemaker potentials produced by ET-1 were 4.1 ± 1 mV

Fig. 6. Effects of ET antagonists on ET-1 induced responses or selective ET_B receptor agonist on pacemaker potentials in cultured ICC of the murine small and large intestine. (A) Pacemaker potentials of ICC in murine small intestine exposed to ET-1 (10 nM) in the presence of ET_A receptor antagonist, BQ-123 (1 μM). BQ-123 blocked the effects of ET-1 on pacemaker potentials. (B) Pacemaker potentials of ICC in murine small intestine exposed to ET-1 (10 nM) in the presence of ET_B receptor antagonist, BQ-788 (1 μM). BQ-788 also blocked the effects of ET-1 on pacemaker potentials. (C) Pacemaker potentials of ICC in murine large intestine exposed to ET-1 (10 nM) in the presence of ET_A receptor antagonist, BQ-123 (1 μM). BQ-123 blocked the effects of ET-1 on pacemaker potentials. (D) Pacemaker potentials of ICC in murine large intestine exposed to ET-1 (10 nM) in the presence of ET_B receptor antagonist, BQ-788 (1 μM). In the presence of BQ-788, ET-1 still caused membrane depolarization and decreased in the frequency and amplitude of pacemaker potentials. (E) Pacemaker potentials of ICC in murine large intestine exposed to the selective ET_B receptor agonist, IRL 1620 (100 nM). The selective ET_B receptor agonist, IRL 1620 did not have any influence on pacemaker potentials. Responses to ET-1 are summarized in (F,G). Bars represent mean values ± S.E. **Asterisks are significantly different from the control (p < 0.01). Dotted lines indicate zero current levels.

(Fig. 6C). However, in the presence of BQ-788, the membrane depolarizations on pacemaker potentials produced by ET-1 were 21.3 ± 2 mV (Fig. 6D). The values of ET_A receptor antagonist, BQ-123 were significantly different from those obtained in controlled conditions (Fig. 6G).

Fig. 7. RT-PCR detection of ET receptor type in single ICC and smooth muscle. Gel electrophoresis of RT-PCR product from single ICC (Aa, Ba) and myocyte (Ab, Bb). RT-PCR product were resolved on 2% agarose gel and visualized by ethidium bromide staining. Size markers were used to indicate the size of the experimental fragments (lane 1). RT-PCR detection of myosin (smooth muscle marker; 195 bp), *c-kit* (ICC marker; 179 bp), protein gene product 9.5 (PGP 9.5, a pan-neural marker; 216 bp), ET_A receptor (159 bp), and ET_B receptor (203 bp) in mRNAs from mouse intestinal single ICC and smooth muscle cell. (A) Single small intestinal ICC (a) and myocyte (b) show RT-PCR fragments for both ET_A (159 bp) and ET_B (203 bp) receptor. (B) Single large intestinal ICC (a) and myocyte (b) show RT-PCR fragments for both ET_A (159 bp) and ET_B (203 bp) receptor.

To verify these results, we also used the selective ET_B receptor agonist, IRL 1620. Under the pacemaker potentials in cultured murine small intestinal ICCs, IRL 1620 caused membrane depolarizations on pacemaker potentials (data not shown). However, under the pacemaker potentials in cultured murine large intestinal ICCs, IRL 1620 did not show any influence on pacemaker potentials (Fig. 6E). These results suggested that ET-1 modulates pacemaker potentials through ET_A and ET_B receptor activation in murine small intestinal ICCs and ET_A receptor activation in murine large intestinal ICCs.

The molecular expression and localization of ET receptor subtypes in ICC

To determine the presence of ET receptor subtypes in murine small intestine, RT-PCR with *c-kit* positive single cell was performed using ET_A and ET_B receptor genespecific primers in ICC. In case of ICC, the expression of ET_A and ET_B was predominant (Fig. 7Aa). Also, in smooth muscle cell, RT-PCR detected transcripts for ET_A and ET_B (Fig. 7Ab). In addition, to determine the presence of ET receptor subtypes in murine large intestine, RT-PCR was performed using ET_A and ET_B receptor genespecific primers. In case of ICC and smooth muscle cell, the expression of ET_A and ET_B was predominant, too (Fig. 7Ba and 7Bb). This finding indicate that both ET_A and ET_B receptor exist in ICC and smooth muscle cell of murine small and large intestine.

Immunohistochemistry for ET_A and ET_B receptors in cultured ICCs

Expression of ET_A and ET_B receptors were investigated by immunohistochemistry on cultured ICCs. Double staining with anti-*c-kit* (a marker of ICC) and anti-ET_A and anti-ET_B receptor antibodies revealed a colocalization of ET_A and ET_B receptor and *c-kit*-like immunoreactivities in cultured ICCs (Fig. 8).

Fig. 8. Expression of ET_A and ET_B protein in cultured ICCs. (A) Double-labelling of ET_A-like immunoreactivity (green) and c-kit-like immunoreactivity (red) on cultured ICCs. The mixed color yellow (arrows) indicates the co-localization of both ET_A-like and c-kit-like immunoreactivity. Scale bars: 20 μ m. (B) Double-labelling of ET_B-like immunoreactivity (green) and c-kit-like immunoreactivity (red) on cultured ICCs. Several c-kit-like immunoreactive cell bodies and fibres are colocalized with ET_B-like immunoreactivity (yellow). Scale bars: 20 μ m. Cultured ICCs show co-localization of ET_A and ET_B and c-kit-like immunoreactivity.

Discussion

In the present study, we first demonstrated that ET induces depolarization of the membrane and inhibits pacemaker potentials in intestinal ICC. These effects are mediated through external Ca²⁺ influx and internal Ca²⁺ release via protein kinase C- or cyclooxygenase-independent mechanism.

ET_A and ET_B are G protein-coupled receptors. ET_A receptors stimulate phosphatidylinositol turnover (1,4,5 inositol-phosphate (IP₃) and diacylglycerol/protein kinase C (DAG/PKC)), while ET_B receptors activate extracellular Ca²⁺ influx via non-L-type membrane channels, resulting in increased intracellular calcium [Ca²⁺]_i. Increased calcium concentrations cause activation of calcium/calmodulin-dependent myosin light-chain kinase (MLCK) and phosphorylation of 20-kDa myosin light chains (MLC), resulting in increased myosin ATPase activity that culminates in smooth muscle contraction [33-36]. In this study, ET induced depolarization of the membrane and inhibition of pacemaker potentials in intestinal ICCs is mediated by the release of 1,4,5-inositol triphosphate (IP₃)-dependent intracellular Ca²⁺ release from endoplasmic reticulum and by external Ca²⁺ influx. ET causes contraction and relaxation of smooth muscle, electrolyte excretion as well as direct neural effects in many physiologic systems including the cardiovascular, respiratory, GI, genitourinary and central nervous system. ET modulates the vascular tone, heart contraction, tone of airways in the lung, electrolyte excretion in the kidney and circulatory as well as respiratory functions in the brain [13, 17, 37-41]. Endothelin receptors are widely expressed in all tissues. Receptors are also localized to non-vascular structures such as epithelial cells as well as occurring in the central nervous system on glial and neurons. Both ET_A and ET_B receptors are widely distributed, particularly in blood vessels [42, 43]. ET_A receptors present on smooth muscle cells are mainly responsible for contraction, but in animals this can vary depending on the species and vessels. Many agonists and antagonists have been developed, although selective ET_A receptor agonists have not been reported to date. In the GI tract, ET causes contraction and/or relaxation of the esophagus, stomach, ileum and colon. ET causes smooth muscle contraction through interaction with ET_A receptors, ET_B receptors or both subtypes, depending on tissues and species [17, 44-50]. Previous studies have shown that ET causes gastrointestinal muscle contraction through interaction with both ET_A and ET_B receptors in

the esophageal muscularis mucosae, gallbladder, ileum, and cecum [44-46]. On the other hand, although the common bile duct muscle and gastric muscle contraction cells possess both ET_A and ET_B receptors, only ET_A receptors mediate contraction [47, 48]. In addition to contraction, ET causes smooth muscle relaxation through interaction with ET_A receptors or ET_B receptors. In the stomach strips, ET_A receptors mediate relaxation [49]; however, in the ileum strips, ET_B receptors mediate relaxation [50]. In this study, in the cultured mouse ICCs, both ET_A and ET_B mediate the contraction of small intestine, however, only ET_A mediates the contraction of large intestine.

ICCs serve as the pacemaker cells of the GI tract. ICCs generated the spontaneous pacemaker potentials and conducted the slow waves into the smooth muscle syncytium because ICCs are electrically coupled to neighboring smooth muscle cells. Smooth muscle cells respond to the slow wave depolarization with the activation of L-type Ca^{2+} channels. Also the smooth muscle response is regulated by neural inputs. Both excitatory and inhibitory enteric motor neurons are closely associated with ICCs. Neural modification of the smooth muscle response, via ICCs, modulates excitation-contraction coupling between slow waves and contractions. Therefore, loss or dysfunction of any part of the ICCs, smooth muscle cells and neurons can result in GI motility disorders [51].

In this study, ET depolarized the pacemaker potential and decreased the frequency and amplitude of pacemaker activity. Generally, if the pacemaker potential in ICCs was depolarized and the frequency and amplitude of pacemaker activity was decreased, ICCs were contracted such as carbachol (CCh) [52]. Therefore, ET could affect the pacemaker activities of ICCs and the consequent contractions of smooth muscle cells. However, the smooth muscle response is also regulated by neural inputs. Therefore, we might consider the ICCs, smooth muscle cells, and neurons on the effect of ET on GI tract. ET might contract or relax on GI tract depending on the animal species, gut segment, the profile of activated receptors and interactions with other mediators acting at target sites [53]. Therefore, we will investigate the effect of ET on ICCs, smooth muscle cells and neurons in mouse intestine in future.

In conclusion, ET-1 modulates pacemaker potentials through ET_A and ET_B receptor activation in murine small intestinal ICCs and ET_A receptor activation in murine large intestinal ICCs by external Ca^{2+} influx and internal Ca^{2+} release via protein kinase C or cyclooxygenase-independent mechanism.

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