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Effects of ATPon Pacemaker Activity in Interstitial Cells of Cajal of Small Intestine

朝鮮大學校大學院

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朝鮮大學校大學院

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朴 一 求의 博士學位論文을認准함

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: 소장 카할사이질 세포의 자발적 활동도에 대한 ATP 효과

박일구

지도교수: 박찬국

조선대학교대학원의학과

ATP는세포내에너지근원이며또한세포밖신호전달매개물질로퓨린성 수용체를통하여다양한생리학적작용을나타낸다. 퓨린성수용체는위장관내에도분포하여위장관운동, 분비및흡수에관여하고있다. ATP는위장관신경계에서분비된후평활근에작용하여수축성을조절하고있다. 카할사이질세포 (Interstitial Cells of Cajal; ICCs)는위장관내향도잡이세포로평활근에서자발적수축을초래하며여러내인성인자들의작용을매개하여위장관운동조절에중요한역할을하고있다. 카할사이질세포

에서도형태학적연구에의해퓨린성수용체가존재하고있으나아직기능적인역할에대한연구는미진한바본연구는세포밖 ATP의카할사이질세포의자발적향도잡이활동도에대한작용을연구한내용으로다음과같은실험결과들을얻었다.

1. ATP는소장카할사이질세포에서향도잡이세포막전압의탈분극을초래하였으며농도-의존적으로긴장성내향성전류 (tonic inward currents)를발생시켰다.
2. ATP에의해서발생되는긴장성내향성전류의발생은세포밖나트륨이온을제거하거나, 비선택성양이온통로차단제인flufenamic acid 또는nifulmic acid에의해서억제되었다.
3. 세포밖칼슘이온을제거하거나내형질세망에서칼슘유입을차단하는thapsigargin를투여한경우 ATP에의해서발생되는긴장성내향성전류의발생이크게억제되거나차단되었다.
4. 세포내칼슘농도측정실험에서 ATP 투여시카할사이질세포내자발적칼슘농도변화의빈도가증가되었다.
5. Protein kinase A 억제제,protein kinase C 억제제, 및tyrosine

kinase 억제제모두ATP에의해서발생되는긴장성내향성전류의발생에는아무런효과를보이지않았다.

이상의실험결과들로부터소장카할사이질세포에서세포밖 ATP는퓨린성수용체를통하여향도잡이활동도의변화를초래하며그기전은세포밖칼슘유입과세포내칼슘분비에의한비선택성양이온통로의활성화를통하여이루어지는것으로생각된다. 따라서 ATP는퓨린성수용체를통하여카할사이질세포에작용한후평활근에작용함으로서위장관운동조절에관여함을나타내며퓨린성수용체가여러위장관운동성질환치료의표적제로서작용할수있음을시사한다.

핵심단어:카할사이질세포, 향도잡이전압, 퓨린성수용체위장관운동,소장

INTRODUCTION

Gastrointestinal (GI) smooth muscles show spontaneous periodic mechanical contractions without neuronal stimulation. Spontaneous contractions of GI smooth muscles depend on electrical cyclic depolarization of membrane potentials and termed as a slow waves. Slow waves determine the frequency and timing of GI smooth muscle contractions and contribute to peristaltic and segmenting motor activity (Szurszewski, 1987). Many enteric neurotransmitters, endogenous substances, and drugs modulate GI motility by changing the frequency and configuration of slow waves (Olsson and Holmgren, 2001). Therefore, slow waves play an important role in regulating GI motility. The cause of slow waves is due to presence of interstitial cells of Cajal (ICCs) (Sanders, 1996; Thomsen et al., 1998). ICCs are specialized enteric cells that generate slow waves by producing spontaneous inward currents, referred as pacemaker currents in GI tract (Koh et al., 1998). They formed the network each other and connected with neighboring smooth muscles through gap junctions in GI tract, thus pacemaker currents directly transmit to smooth muscles using gap junctions (Hanani et al., 2005). In addition to, enteric neuronal varicosities are very closely

associated with ICCs and transduce neuronal signals to smooth muscles (Sanders, 2006). ICCs also act as stretch receptors due to the changes of electrical slow waves by luminal distension (Won et al., 2005). Many articles showed that the disruption of ICCs networks was related with several motility disorders in GI tract, suggesting that ICCs are very important components in GI motility (Vanderwinden and Rumessen, 1999; Jain et al., 2003).

ATP is an intracellular energy source and also acts as an extracellular signal molecule through purinergic receptors. Purinergic receptors are widely distributed in the body and its activation represents the diverse physiological and pathological actions (Burnstock and Kennedy, 2011). Purinergic receptors are classified with two types, P1 receptor and P2 receptor. P1 receptor is sensitive to adenosine, whereas P2 receptor is sensitive to ATP (Ralevic and Burnstock, 1998). Purinergic receptors are also distributed in GI tract and play an important role in modulating motility, secretion and absorption (Giaroni et al., 2002). In GI tract, ATP is released from enteric neurons to act on P2 receptors of smooth muscles and induced relaxation and contraction. ATP also acts on prejunctional nerve terminals and modulated transmitter release thereby involved in the control of smooth muscle activity (Bornstein, 2008). Furthermore, ATP

modulates the enteric synaptic transmission thereby involved the control of mucosal secretion and absorption (Christofi, 2008;Burnstock, 2008). Together with, P2 receptors detected by immunohistochemistry and P2 mRNA are seen in ICCs of small intestine from mouse and guinea-pig (Vanderwinden et al., 2003; Burnstock and Lavin, 2002; Van Nassauw et al., 2006; Chen et al., 2007). These reports suggest that purinergic receptors can modulate the GI motility by acting on ICCs. However, the functional study of purinergic receptors on pacemaker currents of ICCs has not been studied yet. Therefore, I investigated extracellular ATP role on the electrical pacemaker activities in cultured ICCs from the mouse small intestine.

MATERIALS AND METHODS

Ethics

All experiments were performed according to the Guiding Principles for the Care and Use of Animals approved by the Ethics Committee of Chosun University and the National Institute of Health Guide for the Care and Use of Laboratory Animals. Every effort was made to minimize both the number of animals used and the suffering of the animals.

Solutions and drugs

The cells were bathed in a solution containing 5 mmol/L KCl, 135 mmol/L NaCl, 2 mmol/L $CaCl_2$, 10 mmol/L glucose, 1.2 mmol/L $MgCl_2$, and 10 mmol/L HEPES, adjusted to pH 7.2 with Tris. NaCl was replaced with equimolar *N*-methyl-$_D$-glucamine for making Na^+-free solution, and $CaCl_2$ was omitted in the bath solution for Ca^{2+}-free solution. The pipette solution contained 20 mmol/L K-aspartate, 120 mmol/L KCl, 5 mmol/L $MgCl_2$, 2.7 mmol/L K_2ATP, 0.1 mmol/L Na_2GTP, 2.5 mmol/L creatine phosphate disodium, 5 mmol/L HEPES, and 0.1 mmol/L EGTA, adjusted to pH 7.2 with Tris.

The drugs used were ATP, chelerythrine, SQ 22536, genistein, flufenamic acid, nifulmic acid, and thapsigarginfrom Sigma, (San Diego, CA, U.S.A.).

Preparation of cells and tissues

Balb/C mice (3–8 days old) of either sex were anesthetized with ether and were 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. The luminal contents were washed away with Krebs-Ringer bicarbonate solution. The tissues were pinned to the base of a Sylgard dish and the mucosa was removed by sharp dissection. Small strips of intestinal muscle were equilibrated in Ca^{2+}-free Hank's solution containing 5.36 mmol/L KCl, 125 mmol/L NaCl, 0.34 mmol/L NaOH, 0.44 mmol/L Na_2HCO_3, 10 mmol/L glucose, 2.9 mmol/L sucrose, and 11 mmol/L HEPES for 30 min, and the cells were dispersed with an enzyme solution containing 1.3 mg/mL collagenase (Worthington Biochemical Co, Lakewood, NJ, USA), 2 mg/mL bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA), 2 mg/mL trypsin inhibitor (Sigma) and 0.27 mg/mL ATP. Cells were plated onto sterile glass coverslips

coated with poly L-lysine (2.5 g/mL, Sigma) in 35 mm culture dishes. The cells were then cultured at 37°C in a 5% CO_2 incubator in SMGM (smooth muscle growth medium; Clonetics Co., Walkersville, MD, 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 identified immunologically with the use of a monoclonal antibody for kit protein (ACK_2) labeled with Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA).

Patch clamp experiments

The whole-cell configuration of the patch clamp technique was used to record membrane currents (voltage clamp) and membrane potentials (current clamp) from cultured ICC after 2-3 days in culture. We recorded from small clusters of ICC, as spontaneous inward currents from small groups of cells are more robust and regular than from single cells. Currents or potentials were amplified byAxopatch 200B (Axon Instruments, Foster, CA, USA). A command pulse was applied using an IBM compatible personal computer andpClamp software (version 9.2; Axon Instruments). The data were filtered at 5 kHz, and the recorded data

were displayed on a computer monitor saved for data analysis and future references. Results were analyzed using the Clampfit program (Axon Instruments) and Graph Pad Prism (version 2.01) software. All experiments were performed at 30°C.

Measurement of the intracellular Ca^{2+} concentration

Changes in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were monitored by using fluo-3/AM, which was initially dissolved in dimethyl sulfoxide and stored at -20 °C. Cultured ICC on coverslips (25 mm) were rinsed twice with a bath solution (5 mM: KCl, 135 mMNaCl, 2 mM $CaCl_2$, 10 mM glucose, 1.2 mM $MgCl_2$ and 10 mM HEPES, adjusted to pH 7.4 with Tris). The coverslips were then incubated in the bath solution containing 5 μM fluo-3 with 5% CO_2 at 37 °C for 5 min, rinsed two more times with the bath solution, mounted on a perfusion chamber, and were scanned every 0.4 seconds with a Nikon Eclipse TE200 inverted microscope equipped with a Perkin-Elmer Ultraview confocal scanner and a Hamamatsu Orca ER 12-bit CCD camera. (×200). Fluorescence was excited at a wavelength of 488 nm, and emitted light was observed at 515 nm. During scanning of the Ca^{2+} imaging, the temperature of the

perfusion chamber containing the cultured ICC was kept at 30 °C. Variations of the intracellular Ca^{2+} fluorescence emission intensity were expressed as F1/F0, where F0 is the intensity of the first imaging.

Statistical analysis

Data are expressed as the mean ± standard error. Differences in the data were evaluated by use of the Student's t test. *P* values less than 0.05 are considered a statistically significant difference. The *n* values reported in the text refer to the number of cells used in the patch clamp experiments.

RESULTS

Effects of extracellular ATP on pacemaker activities in ICCs

Under the current clamp mode (I = 0), ICC generated spontaneous pacemaker potentials and pacemaker currents in voltage clamping mode (Fig. 1A and B). Exposure to ATP (200 μM) produced depolarization of the resting membrane potential with a decrease of the amplitude of the pacemaker potential (Fig. 2A). Under control conditions with the current clamp mode, resting potentials and the amplitude of pacemaker potential were -58 ± 3 mV and 23 ± 7 mV. In the presence of ATP, the resting membrane potential and the amplitude of pacemaker potentials were -36 ± 2 mV and 5 ± 2 mV, respectively (n = 7, Fig. 2B and C). Under a voltage clamp at a holding potential of -70 mV, exposure to ATP induced tonic inward currents (resting currents) and reduced amplitude of pacemaker currents in a concentration-dependent manner (Fig. 3A-C). The summarized values and a bar graph of the effects of ATP on pacemaker currents are shown in Figure 3D and E (n = 5~6).

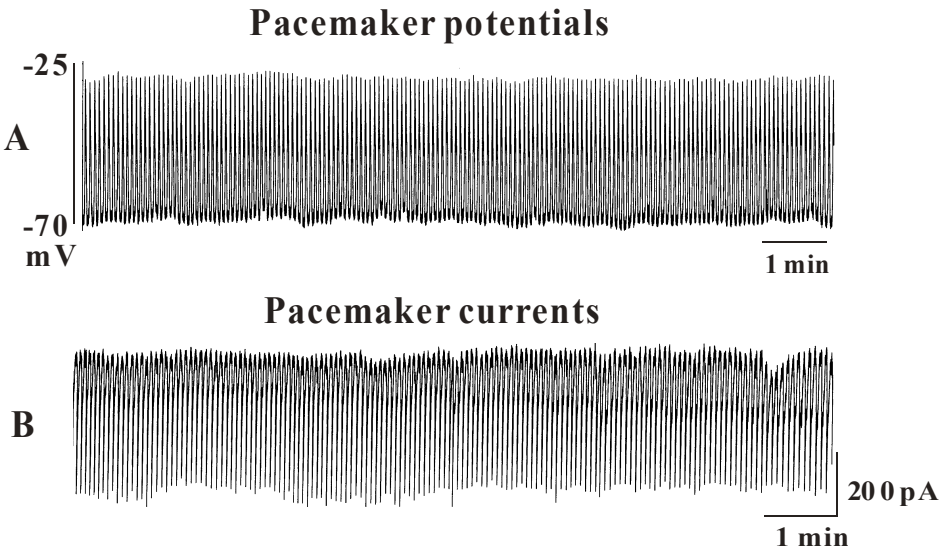

Figure 1. Typical traces of pacemaker potentials in current clamping mode (A) and pacemaker currents in voltage clamping mode at a holding potential of -70 mV (B) in ICCs from mouse small intestine.

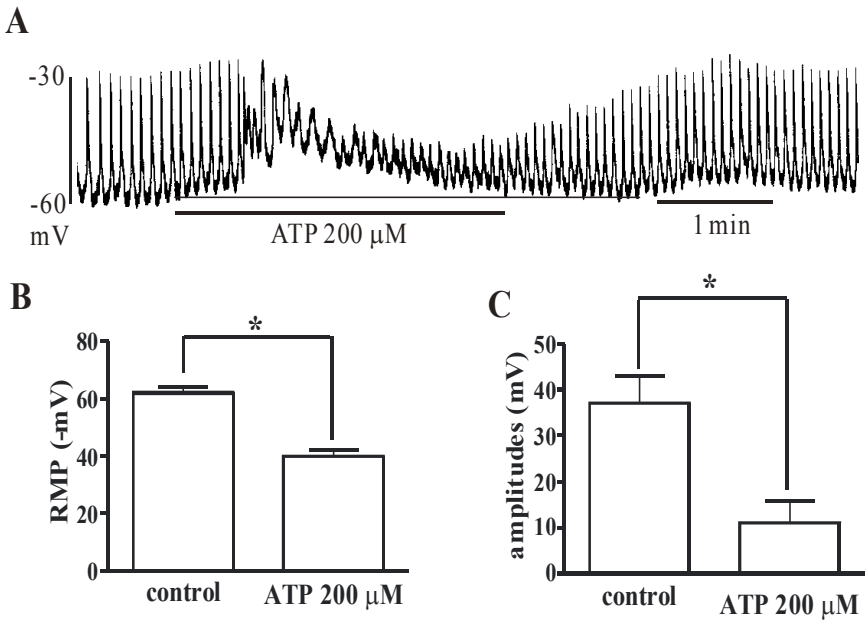

Figure 2. Effects of external ATP on pacemaker potentials of ICCs from mouse small intestine. (A) The treatment with 200 μM of ATP on pacemaker potentials depolarized the membrane with the decrement of amplitude of pacemaker potentials. The effects of ATP on pacemaker potentials were summarized in (B) and (C). Bars represent mean ± SE values. *P < 0.05: significantly different from control. RMP: resting membrane potential.

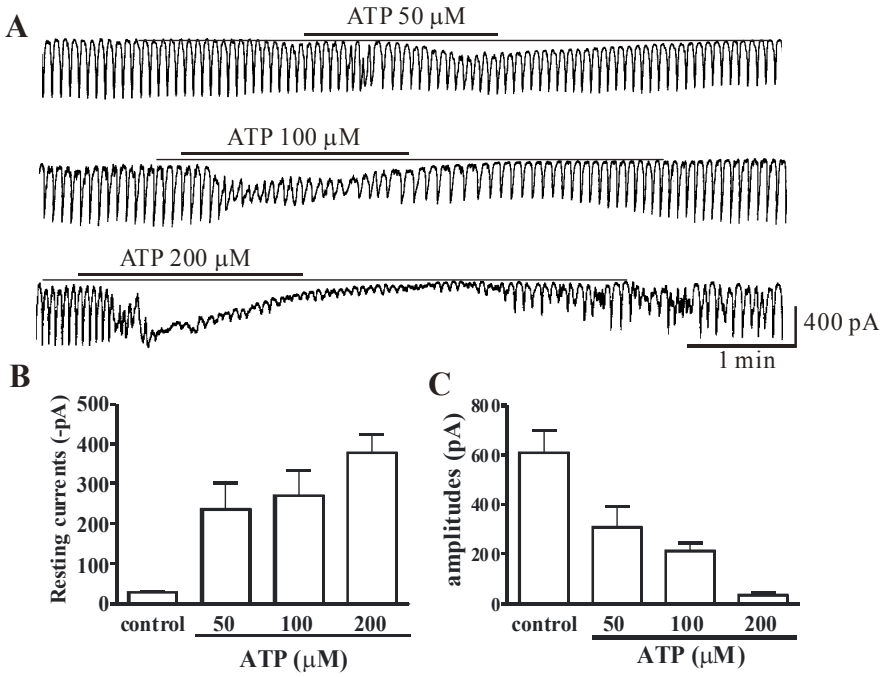

Figure 3. Effects of external ATP on pacemaker currents of ICCs from mouse small intestine. Pacemaker currents of ICCs recorded at a holding potential of -70 mV and exposed to various concentrations of ATP (from 50 to 200 μM). (A) The external ATP produced tonic inward currents and decreased amplitude of pacemaker currents by concentration-dependent manner. Responses to ATP were summarized in (B) and (C). Bars represent mean ± SE values.

Effects of extracellular ATP in the presence of Na^+-free solution and nonselective cation channel blockers

External ATP gated nonselective cation channels through P2 receptors (Jiang et al, 2013). To determine the characteristic of the tonic inward currents induced by ATP, we tested the effects of ATP in the presence of an external Na^+-free solution or non-selective cation channel (NSCC) blockers. Exposure to the external Na^+-free solution abolished the generation of pacemaker currents. Under this condition, the tonic inward currents induced by ATP (200μM) were reduced (Fig. 4A). Under normal conditions, the resting currents of the tonic inward currents induced by ATP was -409 ± 27 pA, and in presence of external Na^+-free solution, the resting currents by was -89 ± 40 pA (n = 5, Fig. 4B). Together, flufenamic acid (10 μM), an inhibitor of NSCC, abolished the generation of pacemaker currents and blocked the ATP-induced tonic inward currents (Fig. 5A). In the presence of flufenamic acid the resting currents of tonic inward currents induced by ATP was -29 ± 8 pA (n = 5, Fig. 5B). Also nifulmic acid, another NSCC inhibitor, suppressed tonic inward currents induced by ATP (Fig. 6A). In the presence of nifulmicacid the

resting currents of tonic inward currents induced by ATP was -40 ± 12 pA (n = 6, Fig. 6B). These results suggest that the ATP-induced tonic inward currents are mediated by NSCCs.

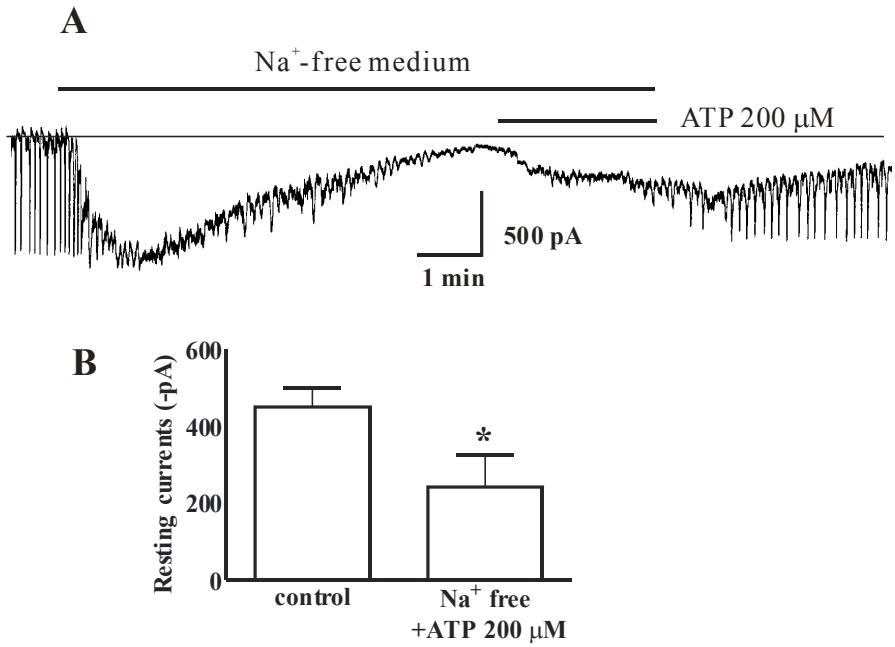

Figure 4. The effects of external Na^+-free solution on the ATP-induced tonic inward currents of ICCs from mouse small intestine. (A) The application of an external Na^+-free solution abolished the generation of pacemaker currents. In this condition, the tonic inward currents induced by ATP (200 μM) were markedly suppressed. Responses to ATP in the external Na^+-free solution were summarized in (B). Bars represent mean ± SE values. *$P < 0.05$: significantly different from control.

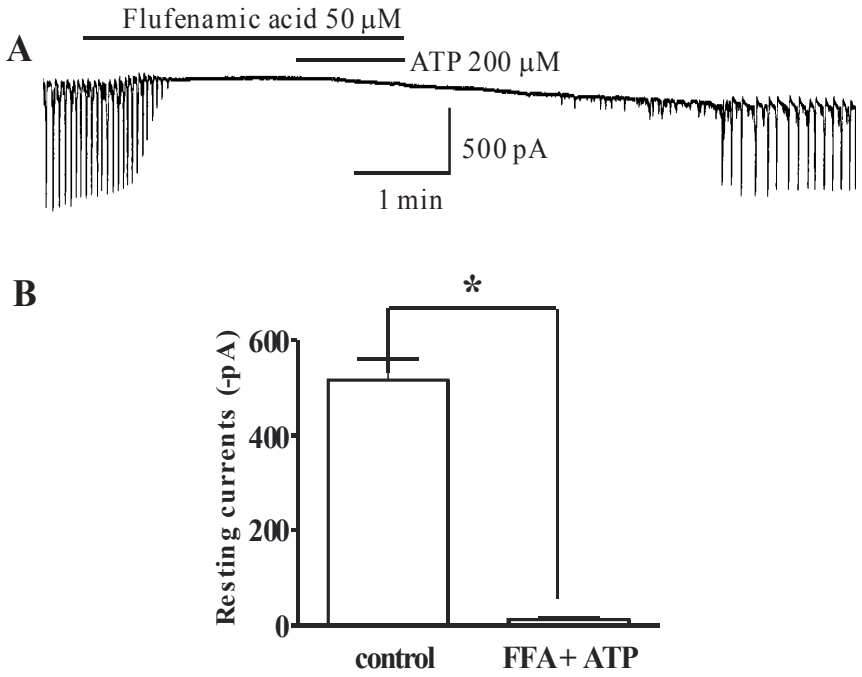

Figure 5. The effects of flufenamic acid on the ATP-induced tonic inward currents of ICCs from mouse small intestine. (A) Flufenamic acid (50 μM), an inhibitor of non-selective cation channels, abolished the generation of pacemaker currents. In this condition, the tonic inward currents induced by ATP (200 μM) were almost blocked. Responses to ATP in the presence of flufenamic acid were summarized in (B). Bars represent mean ± SE values. *P < 0.05: significantly different from control.

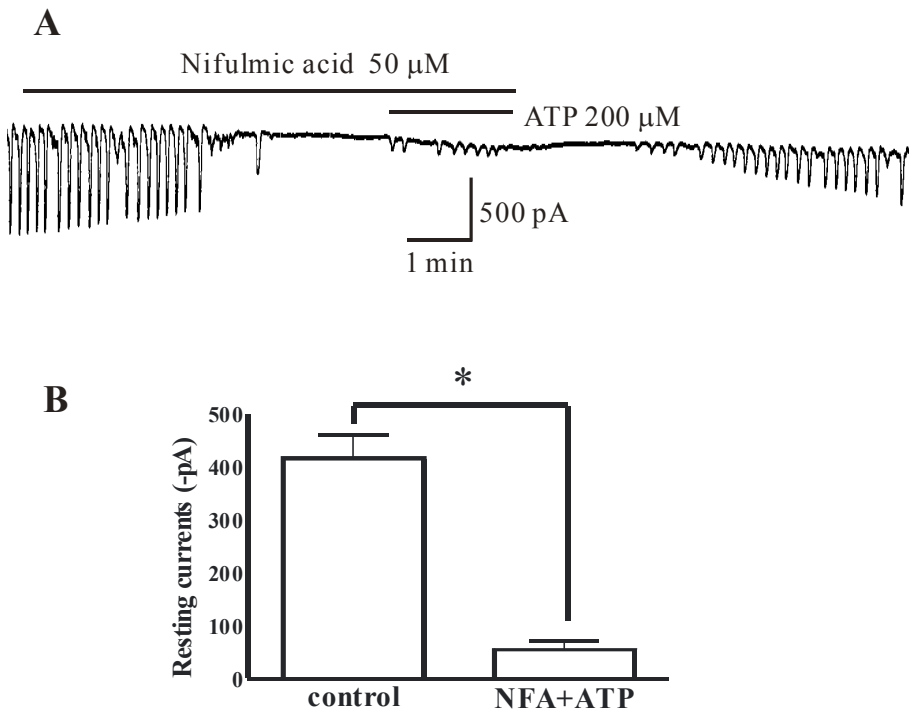

Figure 6. The effects of nifulmic acid on the ATP-induced tonic inward currents of ICCs from mouse small intestine. (A) Nifulmic acid (50 μM), an inhibitor of non-selective cation channels, abolished the generation of pacemaker currents. In this condition, the tonic inward currents induced by ATP (200 μM) were almost blocked. Responses to ATP in the presence of nifulmic acid were summarized in (B). Bars represent mean ± SE values. *P < 0.05: significantly different from control.

Effects of extracellular ATP in the presence of Ca^{2+}-free solution or a Ca^{2+}-ATPase inhibitor from endoplasmic reticulum

To investigate the role of external Ca^{2+} or internal Ca^{2+}, exposure of ICCs to ATP was tested under external Ca^{2+}-free conditions and in the presence of thapsigargin, a Ca^{2+}-ATPase inhibitor of the endoplasmic reticulum. The use of an external Ca^{2+}-free solution completely inhibited the pacemaker currents in the voltage clamp mode at a holding potential of -70 mV. In this condition, ATP (200 μM)-induced tonic inward currents were still evident (n = 5, Fig. 7A). The value of the resting currents with ATPtreatment in the Ca^{2+}-free solution was significantly different when compared with a control value obtained in normal solution (Fig. 7B). The treatment with thapsigargin (5 μM) also inhibited the pacemaker currents of the ICCs and blocked the ATP (200 μM)-induced tonic inward currents (Fig. 8A). In the presence of thapsigargin, the value of the resting currents with ATP treatment with was significantly different from the value obtained with ATP in the absence of thapsigargin (n = 6, Fig. 8B).

Effects of intracellular Ca^{2+} intensity by extracellular ATP in ICCs

As intracellular Ca^{2+} ($[Ca^{2+}]_i$) oscillations in ICCs are considered the primary mechanism for the pacemaker activity in GI activity (Ward *et al*., 2000), we examined the effect of ATP on $[Ca^{2+}]_i$ oscillations in ICCs. In this study, we measured spontaneous $[Ca^{2+}]_i$ oscillations of ICCs that are connected with cell clusters. Spontaneous $[Ca^{2+}]_i$ oscillations were observed in many ICCs (low magnification; data not shown), which were loaded with fluo3-AM and spontaneous regular $[Ca^{2+}]_i$ oscillations were seen in a time series (Fig. 9). Especially, the frequency of $[Ca^{2+}]_i$ oscillation was about 8 – 10 cycles/min. The difference of frequency between $[Ca^{2+}]_i$ oscillation and pacemaker activity is the reason that we scanned every 0.4 seconds for recording of $[Ca^{2+}]_i$. In the presence of ATP (200 μM), the basal points of $[Ca^{2+}]_i$ oscillations increased but the peak points of $[Ca^{2+}]_i$ oscillations were slightly decreased. These results suggest that the action of ATP in ICCs may involve the regulation of spontaneous $[Ca^{2+}]_i$ oscillations.

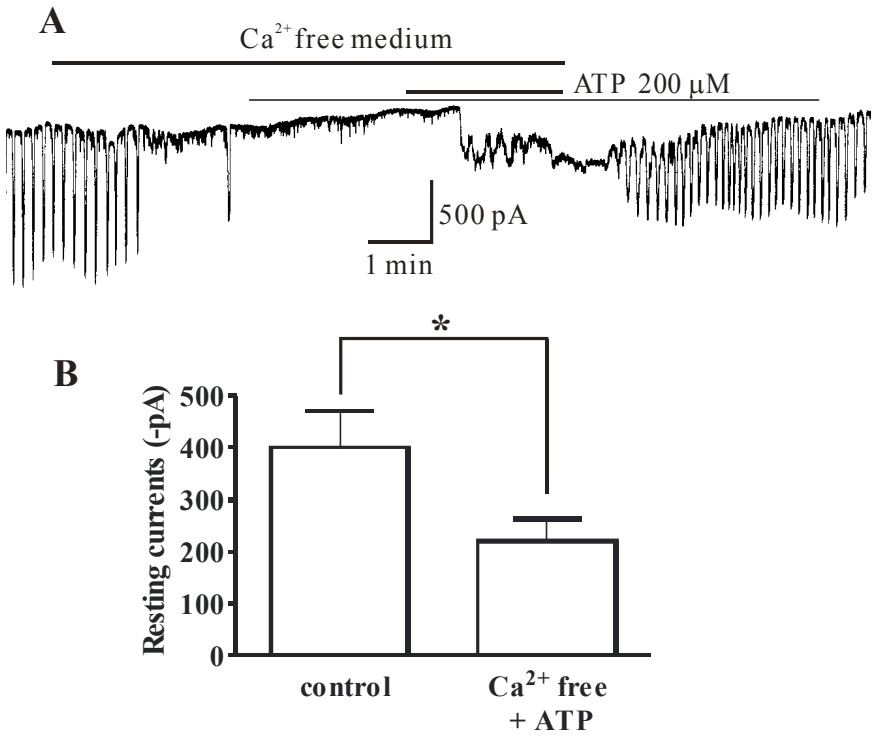

Figure 7. The effects of external Ca^{2+}-free solution on the ATP-induced tonic inward currents of ICCs from mouse small intestine. (A) The application of an external Ca^{2+}-free solution abolished the generation of pacemaker currents. In this condition, the tonic inward currents induced by ATP (200 μM) were markedly suppressed. Responses to ATP in the external Ca^{2+}-free solution were summarized in (B). Bars represent mean ± SE values. *$P < 0.05$: significantly different from control.

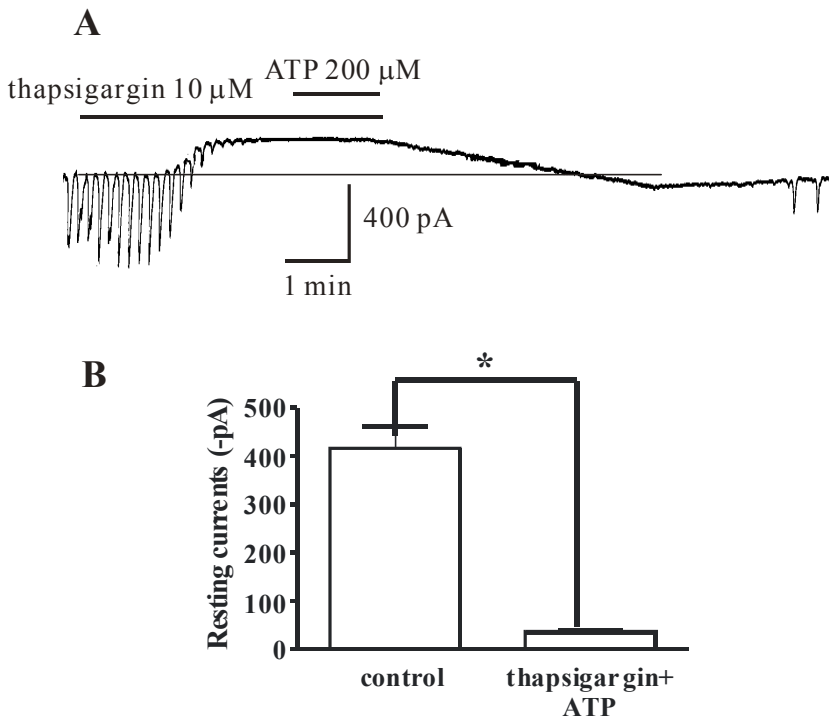

Figure 8. The effects of thapsigargin on the ATP-induced tonic inward currents of ICCs from mouse small intestine. (A) Thapsigargin (10 μM), an inhibitor of Ca^{2+} ATPase in endoplasmic reticulum, abolished the generation of pacemaker currents. In this condition, the tonic inward currents induced by ATP (200 μM) were almost blocked. Responses to ATP in the presence of thapsigargin were summarized in (B). Bars represent mean ± SE values. *P < 0.05: significantly different from control.

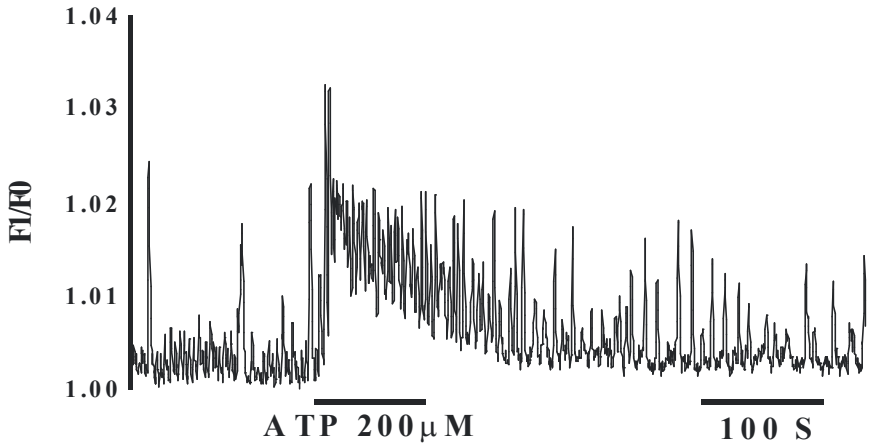

Figure 9. The effects of ATP on intracellular Ca^{2+} in ICCs from mouse small intestine. ATP (200 μM) increased basal level of intracellular Ca^{2+} intensity and increased the Ca^{2+} oscillations.

Effects of extracellular ATP in the presence of adenylate cyclase inhibitor, tyrosine kinase inhibitor and protein kinase inhibitor

The effects of SQ-22536 (an inhibitor of adenylate cyclase), genestein (an inhibitor of tyrosine kinase), and chelerythrine (an inhibitor of protein kinase C) were examined to determine whether the cAMP- or tyrosine kinase-, or protein kinase C (PKC) pathway is involved in the effects of ATP. SQ-22536 (100 μM), genestein (100 μM), or chelerythrine (10 μM) itself had no effects on pacemaker currents. In the presence of SQ-22536, genestein, or chelerythrine, ATP (200 μM)-induced tonic inward currents were still evident (n = 5, Fig. 10 A-C).

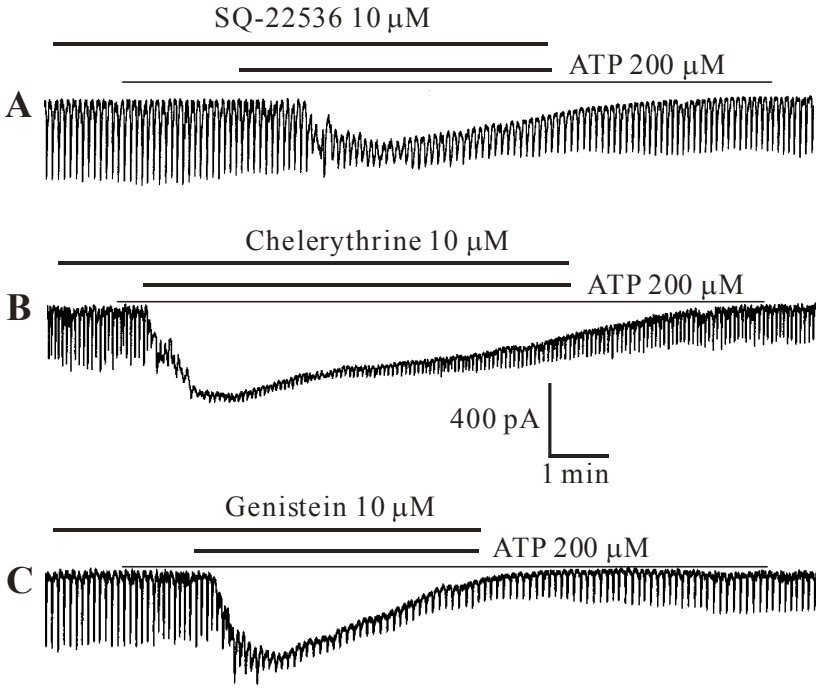

Figure 10. The effects of adenylate cyclase A inhibitor, protein kinase C inhibitor, and tyrosine kinase C inhibitor on the ATP-induced tonic inward currents of ICCs from mouse small intestine. (A) SQ-22536 (200 μM), an adenylate cyclase A inhibitor, (B) chelerythrine (200 μM), a protein kinase C inhibitor, (C) genistein (200 μM), tyrosine kinase C inhibitor, did not block the ATP-induced tonic inward currents.

DISCUSSION

In the present study, external ATP modulated the pacemaker activity of intestinal interstitial cells of Cajal (ICCs) through activating non-selective cation channels (NSCCs) by intracellular Ca^{2+}($[Ca^{2+}]_i$)release-dependent pathway.

Purinergic receptors are playing an important role in regulating GI motility.Purinergic receptors are classified with P1 and P2 receptors. P1 receptors are activated by adenosine and P2 receptors are activated by ATP. Again, P2 receptors are comprised with P2X receptors and P2Y receptors. Generally, it has been reported that P2Y receptors activation by ATP mediated inhibitory motor control through enteric neurons or by directly acting on smooth muscles in GI tract (Gallego et al., 2008; Hwang et al., 2012; Hedlund et al., 1986; Martinez-cutillas et al., 2014). In contrast, it has been reported that P2X receptors activation by ATP mediated excitatory motor control in GI tract (Bian et al., 2003; Matsuo et al., 1997; Bornstein, 2008). ICCs are enteric pacemaker cells that regulate smooth muscle contractility and are also expressed P2X and P2Y receptors including human (Vanderwinden et al., 2003; Burnstock and

Lavin, 2002; Van Nassauw et al., 2006; Chen et al., 292). The generation of spontaneous pacemaker currents of ICCs spread to neighboringsmooth muscles via gap junctions and followed to depolarize the membrane, which leads to smooth muscle contractions by activating voltage-dependent Ca^{2+} channels. In the present study, external ATP depolarized the membrane and produced tonic inward currents in ICCs. These results suggest that ATP has an excitatory motor action by modulating pacemaker activity of intestinal ICCs through P2 receptors. P2X receptors are found in both canineand murine colonic smooth-muscle cells (Giaroni et al., 2002; Monro et al., 2004), whichare contracted by P2X stimulation. Also, P2X receptoractivation modified pacemaker activity in some ICCs (Furuzono et al., 2005),so these receptors might regulate intestinal pacemakers. In contrast to P2Y receptors relaxed GI smooth muscles (Gallego et al., 2008). In addition, recently, it was reported that PDGFRα+ cells (fibroblast-like cells) could transduce purinergic signals. P2Y receptors in PDGFRα+ cells hyperpolarize the membrane through activation of apamin-sensitive Ca^{2+}-activated K^{+} channels, which contribute to smooth muscle relaxation in small intestine (Kurahashi et al., 2014; Peri et al., 2013). Therefore, I think that external ATP-induced depolarization of membrane and tonic inward currents in

intestinal ICCs may be mediated by activation P2X receptors, which involves in intestinal motor patterns. In urethral smooth muscle, external ATP contracted smooth muscle through modulating pacemaker activity of urethral ICCs by P2X receptor activation (Bradley et al., 2010). However, it needs further experiments to investigate P2 receptor subtypes in ICCs.

Intestinal ICCs generate spontaneous pacemaker activity that is mediated by voltage-independent non-selective cation channels (NSCCs) or Ca^{2+}-dependent Cl^- channels (Nakayama et al., 2007). Also, P2X receptors are NSCCs that highly permeable to Ca^{2+} (Jiang et al., 2013; Alvarez et al., 2008; Koshimizu et al., 2000). In the present study, external ATP-induced tonic inward currents on ICCs were suppressed or blocked by the removal of external Na^+ and by the treatment of flufenamic acid or nifulmic acid, NSCC blockers. However, DIDS, an inhibitor of Ca^{2+}-dependent Cl^- channels did not block the external ATP-induced actions on ICCs. In canine colon, P2X receptor activation depolarized and excited smooth muscles via the activation of NSCCs (Monaghan, 2006). Also, carbachol showed the similar effects with ATP in intestinal ICCs. Carbachol depolarized the membrane and produced tonic inward currents by activating NSCCs in ICCs and carbachol-induced effects were blocked by the removal of external Na^+ and by the treatment of

flufenamic acid or nifulmic acid (So et al., 2009). Based on these results, I think that external ATP–induced tonic inward currents in intestinal ICCs are mediated by the activation of NSCCs.

The spontaneous pacemaker activity of intestinal ICCs is dependent on extracellular Ca^{2+} and release of $[Ca^{2+}]_i$ from endoplasmic reticulum. Particularly, IP_3-dependnt intracellular Ca^{2+} oscillations are primary pacemaking mechanism to generate pacemaker activity and are well matched periodic activation of pacemaker channels (Ward et al., 2000). Furzono et al. (2005) reported that external ATP modulated $[Ca^{2+}]_i$ activity by P2X receptors in intestinal ICCs from mouse. In the present study, external ATP-induced tonic inward currents were markedly suppressed by the removal extracellular Ca^{2+} or the treatment of thapsigargin, a Ca^{2+} uptake inhibitor of endoplasmic reticulum. Also, in Ca^{2+}-imaging, external ATP increased $[Ca^{2+}]_i$oscillations. In renal vascular myocytes, Ca^{2+}-entry following P2X receptor activation induced IP_3-dependent Ca^{2+} release from endoplasmic reticulum (Povstyan et al., 2011). Thus, it seems that external ATP-induced action on intestinal ICCs is mediated by extracellular Ca^{2+} influx through NSCCs and $[Ca^{2+}]_i$release from endoplasmic reticulum.

cAMP-dependent protein kinase A, tyrosine kinase, or protein kinase C

participate in the process of signal transduction of purinergic receptor activation in several tissues (Han et al., 2005; Brown et al., 2007; Oguma et al., 2007). However, in the present study, these enzyme inhibitors all did not block the external ATP-induced tonic inward currents. Therefore, it seems that other signal transduction mechanisms are involved in intestinal ICCs.

In conclusion, external ATP depolarized the membrane and induced tonic inward currents in intestinal ICCs. These effects were mediated by activating NSCCs through extracellular Ca^{2+} influx and$[Ca^{2+}]_i$release. Thus, it seems that external ATP can regulate smooth muscle activities indirectly by acting on ICCs and can be therapeutic targets for altered GI motility disorders.

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