

Involvement of Thromboxane A₂ in the Modulation of Pacemaker Activity of Interstitial Cells of Cajal of Mouse Intestine

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Although many studies show that thromboxane A₂ (TXA₂) has the action of gastrointestinal (GI) motility using GI muscle cells and tissue, there are no reports on the effects of TXA₂ on interstitial cells of Cajal (ICC) that function as pacemaker cells in GI tract. So, we studied the modulation of pacemaker activities by TXA₂ in ICC with whole cell patch-clamp technique. Externally applied TXA₂ (5 μM) produced membrane depolarization in current-clamp mode and increased tonic inward pacemaker currents in voltage-clamp mode. The tonic inward currents by TXA₂ were inhibited by intracellular application of GDP-β-S. The pretreatment of ICC with Ca²⁺ free solution and thapsigargin, a Ca²⁺-ATPase inhibitor in endoplasmic reticulum, abolished the generation of pacemaker currents and suppressed the TXA₂-induced tonic inward currents. However, chelerythrine or calphostin C, protein kinase C inhibitors, did not block the TXA₂-induced effects on pacemaker currents. These results suggest that TXA₂ can regulate intestinal motility through the modulation of ICC pacemaker activities. This modulation of pacemaker activities by TXA₂ may occur by the activation of G protein and PKC independent pathway via extra and intracellular Ca²⁺ modulation.

Key Words: Thromboxane A₂ (TXA₂), Interstitial cells of Cajal (ICC), Pacemaker currents, Intestinal motility

INTRODUCTION

The prostanoids are oxygenated derivatives of C₂₀ fatty acids, principally arachidonic acid. Five pathways of arachidonic acid metabolism are currently recognized, and prostanoids are the products of the first of these to be discovered, the cyclooxygenase pathway. There are two major classes of prostanoids, the prostaglandins (PGs) and the thromboxanes (Needleman et al, 1986).

PGs have been shown to be widely distributed in the gastrointestinal (GI) tract. Both the mucosa and the muscle layer of the gut are capable of generating the major products of biotransformation of arachidonic acid via the cyclooxygenase pathway such as PGE₂, PGF_{2α}, PGI₂, and thromboxanes (Bennet et al, 1968; Ferreira et al, 1976; LeDuc & Needleman, 1979; Robert, 1981; Sanders, 1981; Sanders, 1984). Among the rest, thromboxane A₂ (TXA₂) is well known to alter the GI activity. For example, TXA₂ was reported to be a potent constrictor of human stomach, ileum and colon (Bennett et al, 1981) and of the rat gastric fundus (Bennett & Sanger, 1982). However, it failed to contract guinea-pig ileum (Coleman et al, 1981). While TXA₂ induced the contraction or relaxation in mouse ileum, suggesting some species difference (Okada et al, 2000).

Interstitial cells of Cajal (ICC) in most locations form gap junctions with muscle cells and also with each other (Daniel & Posey-Daniel, 1984; Zhou & Komuro, 1992; Berezin et al, 1994). Indirect evidence suggests them, to be responsible for the generation of phasic contractions in GI tract (Thuneberg, 1982; Sanders, 1992). ICC generate rhythmic oscillations in membrane potential known as pacemaker potentials and this generation is due to spontaneous inward currents called pacemaker currents (Koh et al, 1998; Thomsen et al, 1998). ICC also form close contacts with nerve terminals (Berezin et al, 1988; Torihashi et al, 1993) and express the receptors for, and respond to, a variety of neurotransmitters (Publicover et al, 1992; Shuttleworth et al, 1993; Young et al, 1993; Sternini et al, 1995; Portbury et al, 1996). Consequently, ICC have also been proposed to act as intermediaries in transmission between neurons and muscle cells (Thuneberg, 1982; Sanders, 1992). Recent evidence suggests that ICC may serve both functions.

There are many reports to indicate that TXA₂ has function on intestinal motility by acting on smooth muscles, however no studies have so far been performed to determine the effects of TXA₂ on electrical events in mouse ICC. Therefore, the purpose of our study was to investigate the effects of TXA₂ on pacemaker activity in cultured ICC.

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ABBREVIATIONS: GDP-β-S, guanosine 5'-[β-thio]diphosphate tri-
thium; ICC, interstitial cells of Cajal.

METHODS

Preparation of cells and tissues

Balb/C mice (3~7 days old) of either sex were anesthetized with ether and sacrificed by cervical dislocation. The small intestines from 1 cm below the pyloric ring to the cecum were removed and opened along the mesenteric border. 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 stripes of intestinal muscle were equilibrated in Ca^{2+} -free Hank's solution 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 murine collagen (2.5 μ g/ml, Falcon/BD) in 35 mm culture dishes. The cells were then cultured at 37°C in a 95 % O_2 -5 % CO_2 incubator in SMGM (smooth muscle growth medium, Clonetics Corp., San Diego, CA, USA) supplemented with 2% antibiotics/antimycotics (Gibco, Grand Island, NY, USA) and murine

stem cell factor (SCF, 5 ng/ml, Sigma). Interstitial cells of Cajal (ICC) were identified immunologically with a monoclonal antibody for Kit protein (ACK₂) labelled with Alexa Fluor 488 (molecular Probe, 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. Currents or potentials were amplified by use of an Axopatch 1-D (Axon Instruments, Foster, CA, USA). Command pulse was applied using an IBM-compatible personal computer and pClamp software (version 6.1; Axon Instruments). The data were filtered at 5 kHz and displayed on an oscilloscope, a computer monitor, and a pen recorder (Gould 2200, Gould, Valley view, OH, USA).

Results were analyzed using pClamp and Sigma plot (version 9.0) software. All experiments were performed at 30°C.

Solutions and drugs

The cells were bathed in a solution containing (mM): 5 mM KCl, 135 mM NaCl, 2 mM $CaCl_2$, 10 mM glucose, 1.2 mM $MgCl_2$, and 10 mM HEPES, pH adjusted to 7.2 with

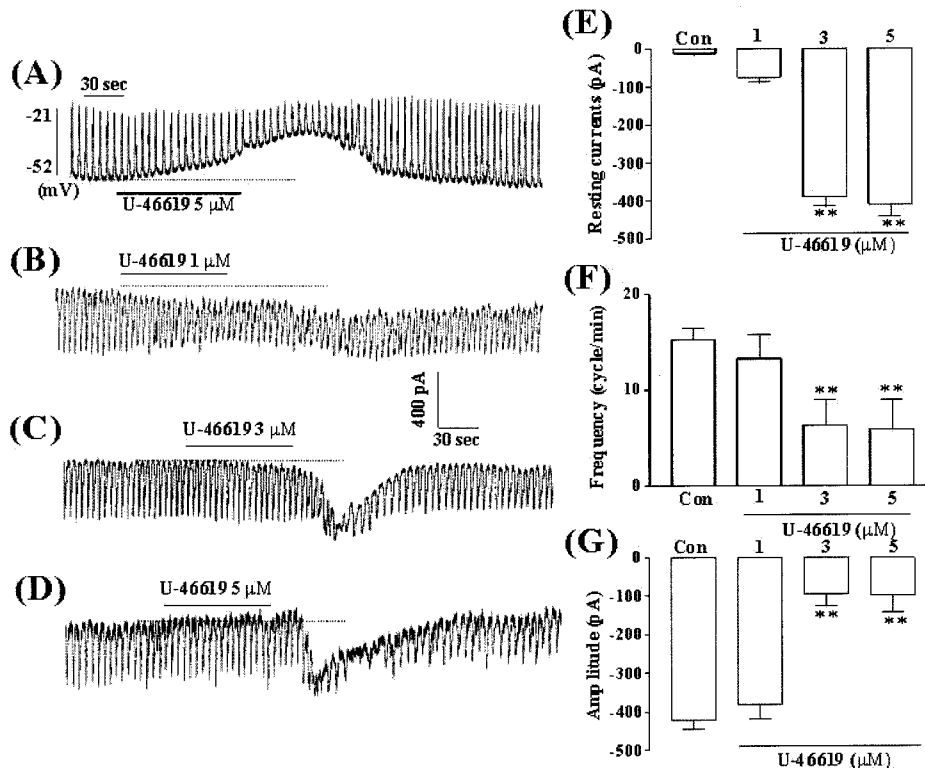


Fig. 1. The effects of TXA₂ on pacemaker potentials and pacemaker currents recorded in cultured ICC from mouse small intestine (A) Pacemaker potentials of ICC which were exposed to TXA₂ (5 μ M) in the current-clamping mode ($I=0$). Vertical solid line scales amplitude of pacemaker potential and horizontal solid line scales for duration of recording (s) pacemaker potentials. (B), (C), and (D) Pacemaker currents of ICC recorded at a holding potential of -70 mV, when exposed to various concentrations of TXA₂ (1, 3, and 5 μ M). The dotted lines indicate zero current levels. Vertical solid line scales amplitude of pacemaker current and horizontal solid line scales duration of recording (s) pacemaker currents. The responses to TXA₂ are summarized in (E), (F) and (G). The bars represent mean values \pm SE. **Significantly different from the untreated control (Con) (p < 0.01).

Tris. The pipette solution contained 140 mM KCl, 5 mM MgCl₂, 2.7 mM K₂ATP, 0.1 mM Na₂GTP, 2.5 mM creatine phosphate disodium, 5 mM HEPES, 0.1 mM EGTA, pH adjusted to 7.2 with Tris.

Drugs used were: U-46619, guanosine 5'-[thio]diphosphate trillithium salt (GDP-β-S), calphostin C, chelethrine, and thapsigargin. All drugs were purchased from Sigma Chemical Co (St. Louis, MO, USA).

Statistical analysis

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

RESULTS

Effect of TXA₂ on pacemaker potentials and currents in cultured ICC

ICC were identified with immunofluorescence using Kit antibody, and had a distinctive morphology easily recognizable in cultures. We performed the electrophysiological recording from cultured ICC under current (*I*=0) and voltage clamp mode. Under current clamp mode, ICC spontaneously showed pacemaker potentials. The resting membrane potential was -48 ± 3 mV and amplitude was 25 ± 6 mV. In the presence of U46619 (5 μM), a thromboxane receptor agonist, membrane potentials were depolarized to -19 ± 2.4 mV, and the amplitude of pacemaker potentials was decreased to 4.7 ± 1.4 mV (*n*=5, Fig. 1A, bar graph not shown). These results suggested that ICC have the spontaneous pacemaker activity, and that U46619 has an effect on this electrical activity of ICC. Under a voltage clamp at a holding potential of -70 mV, the ICC generated spontaneous inward currents. Treatment of cultured ICC with various concentrations of U46619 (1, 3, and 5 μM) produced tonic inward currents and decreased the frequency and the amplitude of pacemaker currents in a dose-dependent manner (Fig. 1B, C, and D). As shown in Fig 1E, F, and G, the values of frequency, amplitude and resting currents about pacemaker currents in control condition were significantly different from those obtained in the presence of U46619 (3 and 5 μM).

Involvement of G proteins in the TXA₂- induced tonic inward currents in cultured ICC

The effects of GDP-β-S, a nonhydrolysable guanosine 5'-diphosphate analogue which permanently inactivates GTP binding proteins, were examined to determine whether the G-protein is involved in the effects of TXA₂ on ICC. When GDP-β-S (1 mM) was in the pipette, U46619 (5 μM) did not show the tonic inward currents (Fig. 2A). In the presence of GDP-β-S in the pipette, the resting currents in control were -19 ± 8 pA. The resting currents by treatment with U46619 in the presence of GDP-β-S were -22.6 ± 5 pA (*n* = 4, Fig. 2B), which were significantly different from those obtained by treatment with U46619 in the absence of GDP-β-S.

External Ca²⁺-free solution and Ca²⁺-ATPase inhibitor of endoplasmic reticulum suppress TXA₂ effects in cultured ICC

To investigate the role of external Ca²⁺ or internal Ca²⁺, TXA₂ was tested under external Ca²⁺-free conditions and in the presence of thapsigargin, a Ca²⁺-ATPase inhibitor of endoplasmic reticulum. The application of external Ca²⁺-free solution completely inhibited the pacemaker currents in voltage clamp mode at a holding potential of -70 mV, and U46619 (5 μM)-induced effects on pacemaker currents in this condition were blocked (*n*=5, Fig. 3A). The value of resting currents with U46619 (5 μM) in Ca²⁺-free solution was significantly different, compared with control value obtained in normal solution (Fig. 3B). In addition, the treatment of thapsigargin (5 μM) inhibited the pacemaker currents in ICC and blocked the U46619-induced tonic inward currents (Fig. 3C). In the presence of thapsigargin, the value of resting currents by treatment with U46619 was significantly different from those obtained by treatment with U46619 in the absence of thapsigargin (*n*=6, Fig. 3D).

Effects of protein kinase C inhibitor on TXA₂-induced responses in cultured ICC

We tested the effects of chelerythrine or calphostin C, an inhibitor of protein kinase C (PKC), to investigate whether TXA₂-induced responses to pacemaker currents are mediated by the activation of PKC. Chelerythrine (1 μM) or calphostin

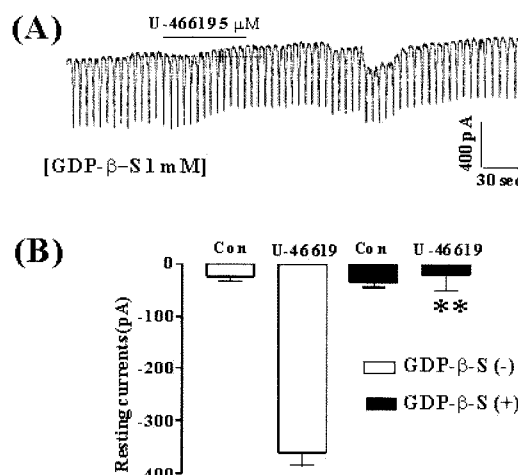


Fig. 2. The effects of GDP-β-S in response to TXA₂ induced pacemaker currents from ICC of mouse small intestine (A) Pacemaker currents from ICC exposed to TXA₂ (5 μM) in the presence of GDP-β-S (1 mM) in the pipette. The tonic inward currents with suppressed amplitudes and frequency induced by TXA₂ were blocked by internally applied GDP-β-S (1 mM). The dotted lines indicate the zero current levels. The effects of TXA₂ in the presence of GDP-β-S are summarized in (B). Vertical solid line scales amplitude of pacemaker current and horizontal solid line scales for duration of recording (s) pacemaker currents. Bars represent mean values ± SE. The effects of GDP-β-S on TXA₂-induced pacemaker currents were significantly different from the TXA₂-induced pacemaker currents (*p* < 0.01). The bars represent mean values±SE. **Significantly different from the untreated control (Con) (*p* < 0.01).

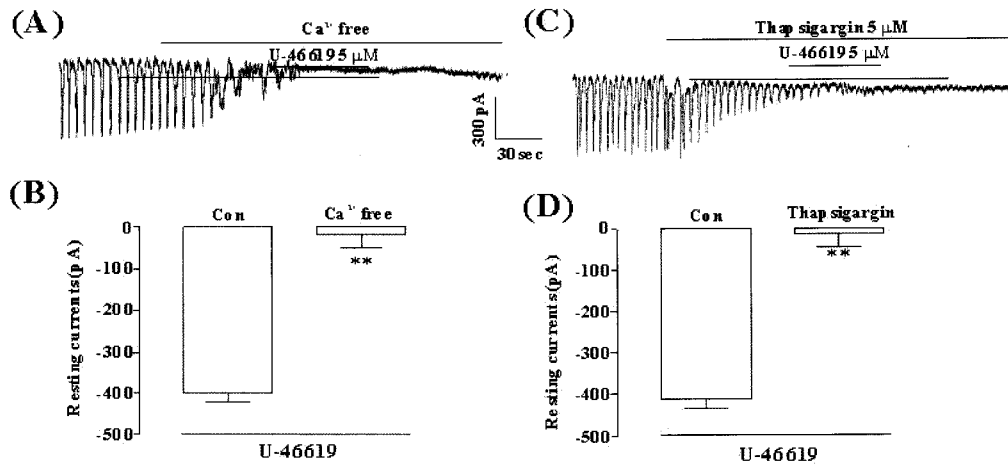


Fig. 3. The effects of an external Ca^{2+} -free solution or thapsigargin on the TXA_2 -induced response on pacemaker currents in cultured ICC from mouse small intestine (A) External Ca^{2+} -free solution abolished the generation of pacemaker currents. Under this condition, the TXA_2 ($5 \mu M$)-induced tonic inward currents were blocked. (C) Thapsigargin ($5 \mu M$) abolished the generation of pacemaker currents. Thapsigargin also blocked the TXA_2 ($5 \mu M$)-induced tonic inward currents. The dotted lines indicate the zero current levels. Responses to the TXA_2 in the external Ca^{2+} -free solution and in the presence of thapsigargin are summarized in (B) and (D). Vertical solid line scales amplitude of pacemaker current and horizontal solid line scales duration of recording (s) pacemaker current. The bars represent mean values \pm SE. **Significantly different from the untreated Control (Con) ($p < 0.01$).

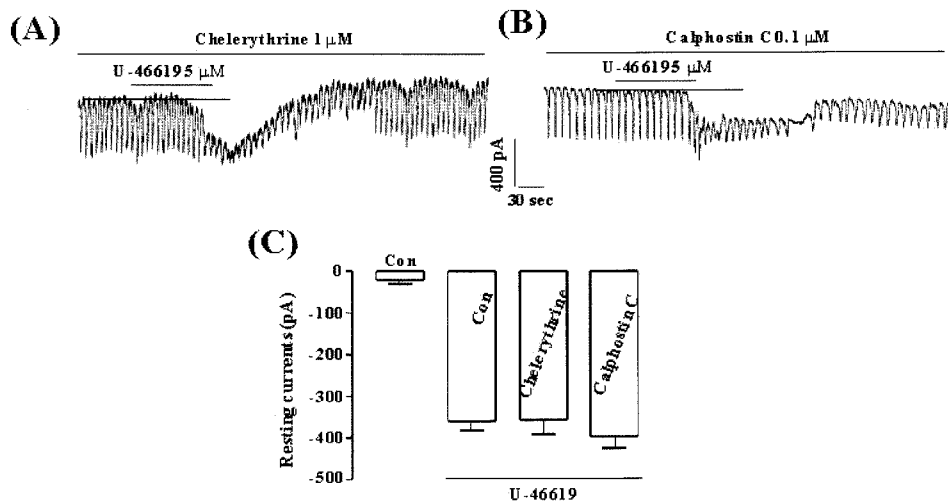


Fig. 4. The effects of chelerythrine or calphostin C on the TXA_2 -induced response on pacemaker currents in cultured ICC from mouse small intestine (A), (B) Pacemaker currents of ICC exposed to TXA_2 ($10 \mu M$) in the presence of chelerythrine ($1 \mu M$) or calphostin C ($0.1 \mu M$). In this condition, the TXA_2 caused tonic inward currents. The dotted lines indicate the zero current levels. Responses to the TXA_2 in the presence of chelerythrine or calphostin C are summarized in (C). Vertical solid line scales amplitude of pacemaker current and horizontal solid line scales duration of recording (s) pacemaker current. The bars represent mean values \pm SE.

C ($0.1 \mu M$) did not have an effect on tonic inward currents by U46619 ($5 \mu M$) (Fig. 4A and C), and the value was not also significantly different, compared with the tonic inward currents by U46619 obtained in the absence of chelerythrine or calphostin C ($n=5$, Fig. 4B and D).

DISCUSSION

Although the effects TXA_2 on the intestinal motility in

tissue and smooth muscle cells have amply been demonstrated, this is the first study to determine the effects of TXA_2 on electrical activity in small intestine.

Present results demonstrate that TXA_2 regulates intestinal motility by modulating the pacemaker currents of ICC, and that this modulation is mediated via acting on G protein and extra- and intracellular Ca^{2+} mobilization in a PKC-independent manner.

While contractile actions of TXA_2 on airway and vascular smooth muscles are well known, those on smooth muscle

of gastrointestinal tract have not yet been fully characterized. Also, TXA₂ have some species difference in GI tract. Recently, it was suggested that TXA₂ has strong contractile actions on both the fundus and ileum, which were mediated mainly via the thromboxane receptors in mice (Okada, 2000). Moreover, the above study showed that TXA₂ has the highest potency among the prostaglandin E₂, D₂, I₂, and F_{2 α} , suggesting physiological or pathophysiological roles of TXA₂ played in the direct or indirect actions on gastrointestinal smooth muscles, which has largely been attributed to its action on vasculature and blood platelets. Namely, the above study indicates the possibility that TXA₂ may have stimulatory or inhibitory functions on electrical activity of ICC. In the present study, we observed that TXA₂ evoked the depolarization of pacemaker potentials and tonic inward currents of pacemaker currents in ICC, implying that the regulation of electrical activity in ICC may be involved in the contractile effects of TXA₂ in intestinal tract.

TXA₂ induces the effects by increasing the cytosolic Ca²⁺ concentration which is independent of extracellular Ca²⁺ and does not involve changes in inositol (1,4,5) trisphosphate (IP₃) levels, suggesting that this TXA₂ action involves mobilization of Ca²⁺ from intracellular stores via an IP₃-independent process in platelet (Nakano et al, 1988; Arita et al, 1989). And many reports suggested that TXA₂ leads to phospholipase C (PLC) activation, indicating that TXA₂ receptors are coupled in part to a pertussis toxin-insensitive G protein (Pollock et al, 1984; Watson et al, 1985; Brass et al, 1987; Sage & Rink, 1987; Siess et al, 1986; Shenker et al, 1991). Overall, these studies suggest that TXA₂ receptors couple directly to G protein, resulting in PLC activation, IP₃-dependent Ca²⁺ mobilization, and activation of PKC activation through diglyceride formation, further coupling TXA₂ receptors to a second G protein. It is not clear whether coupling to the G protein is involved in IP₃-independent Ca²⁺ mobilization and/or a Ca²⁺ channel that mediates the entry of extracellular Ca²⁺. In this study, the tonic inward currents of pacemaker currents were blocked by TXA₂, when GDP β S was present in the pipette. This means that the effects of TXA₂ on electrical activity in ICC may be related with G proteins. Because many suggestions have been made that TXA₂ may have biological activity through mobilization of intracellular Ca²⁺, we used thapsigargin, a potent endoplasmic reticulum Ca²⁺-ATPase inhibitor, and found that those inhibitors suppressed the TXA₂-induced tonic inward currents. These results strongly suggest that the release of Ca²⁺ from internal storage by TXA₂ is essential to produce tonic inward currents, in correspondence with previous suggestions. In addition, it is well known that the generation of pacemaker currents is dependent upon intracellular Ca²⁺ oscillation and the periodic release of Ca²⁺ from endoplasmic reticulum is essential for generation of pacemaker currents. Also, in the present study, chelerythrine or calphostin C, specific and potent PKC inhibitors, did not block TXA₂-induced effects, suggesting that PKC is not involved on the actions of TXA₂ in ICC.

In conclusion, this study describes the effects of TXA₂ on ICC in the mouse small intestine. TXA₂ depolarized the membrane with increased tonic inward currents, which was via external Ca²⁺ influx and internal Ca²⁺ mobilization in a PKC-independent manner. Thus, the action of TXA₂ on ICC may explain the excitatory action of TXA₂ in GI motility.

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