

Bradykinin modulates pacemaker currents through bradykinin B2 receptors in cultured interstitial cells of Cajal from the murine small intestine

¹Seok Choi, ¹Do Young Park, ¹Cheol Ho Yeum, ²In Youb Chang, ³Ho Jin You, ⁴Chan Guk Park, ⁴Man Yoo Kim, ⁵In Deok Kong, ⁶Insuk So, ⁶Ki Whan Kim & ^{*1}Jae Yeoul Jun

¹Department of Physiology, College of Medicine, Chosun University, 375 Seosuk-Dong, Dong-ku, Gwangju 501-759, Korea;

²Department of Anatomy, College of Medicine, Chosun University, 375 Seosuk-Dong, Dong-ku, Gwangju 501-759, Korea;

³Department of Pharmacology, College of Medicine, Chosun University, 375 Seosuk-Dong, Dong-ku, Gwangju 501-759, Korea;

⁴Department of Internal Medicine, College of Medicine, Chosun University, 375 Seosuk-Dong, Dong-ku, Gwangju 501-759, Korea;

⁵Department Physiology, Wonju College of Medicine, Yonsei University, Wonju 220-701, Korea and ⁶Department of Physiology and Biophysics, College of Medicine, Seoul National University, Seoul 110-799, Korea

1 We studied the modulation of pacemaker activities by bradykinin in cultured interstitial cells of Cajal (ICC) from murine small intestine with the whole-cell patch-clamp technique. Externally applied bradykinin produced membrane depolarization in the current-clamp mode and increased tonic inward pacemaker currents in the voltage-clamp mode.

2 Pretreatment with bradykinin B1 antagonist did not block the bradykinin-induced effects on pacemaker currents. However, pretreatment with bradykinin B2 antagonist selectively blocked the bradykinin-induced effects. Also, only externally applied selective bradykinin B2 receptor agonist produced tonic inward pacemaker currents and ICC revealed a colocalization of the bradykinin B2 receptor and c-kit immunoreactivities, but bradykinin B1 receptors did not localize in ICC.

3 External Na⁺-free solution abolished the generation of pacemaker currents and inhibited the bradykinin-induced tonic inward current. However, a Cl⁻ channel blocker (DIDS) did not block the bradykinin-induced tonic inward current.

4 The pretreatment with Ca²⁺-free solution and thapsigargin, a Ca²⁺-ATPase inhibitor in endoplasmic reticulum, abolished the generation of pacemaker currents and suppressed the bradykinin-induced action.

5 Chelerythrine and calphostin C, protein kinase C inhibitors or naproxen, an inhibitor of cyclooxygenase, did not block the bradykinin-induced effects on pacemaker currents.

6 These results suggest that bradykinin modulates the pacemaker activities through bradykinin B2 receptor activation in ICC by external Ca²⁺ influx and internal Ca²⁺ release *via* protein kinase C- or cyclooxygenase-independent mechanism. Therefore, the ICC are targets for bradykinin and their interaction can affect intestinal motility.

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Abbreviations: DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; ICC, interstitial cells of Cajal

Introduction

Bradykinin is not a neuropeptide of the enteric nervous system but rather acts as a local hormone that is composed of nine amino-acid peptides formed from the plasma precursor kininogen during inflammation or tissue injury (Regoli & Barabe, 1980). Bradykinin binds to specific membrane receptors and participates in a variety of physiological and inflammatory reactions throughout the gastrointestinal (GI) system, including visceral pain, increase in intestinal secretion and modulation of enteric neurons (Cuthbert *et al.*, 1984; Gaginella & Kachur, 1989; Julia *et al.*, 1995; Hu *et al.*, 2003). Bradykinin also has a motor effect on the GI tract. Bradykinin either contracts or relaxes GI smooth muscles through

bradykinin 1 (B1) or bradykinin 2 (B2) receptor activation *via* multiple intracellular signal transduction pathways (Calixto & Medeiros, 1991; Feres *et al.*, 1992; Hyman *et al.*, 1992; Rangachari *et al.*, 1993; Hasler *et al.*, 1995; Zagorodnyuk *et al.*, 1998). The motor action of GI smooth muscle is initiated by periodic membrane depolarization, referred to as slow waves. Slow waves play an important role in regulating GI motility by determining the frequency and the timing of smooth muscle contraction (Szurszewski, 1987). Interstitial cells of Cajal (ICC) are pacemaker cells that generate slow waves by producing spontaneous inward currents (pacemaker currents) (Koh *et al.*, 1998; Thomsen *et al.*, 1998). ICC are connected to each other, form a network and form gap junctions with smooth muscle. Pacemaker currents that result from ICC are directly transmitted to

*Author for correspondence; E-mail: jyjun@chosun.ac.kr

smooth muscle through gap junctions (Sanders *et al.*, 1999). In addition, ICC mediate inhibitory and excitatory signals from the enteric nervous system to smooth muscle (Berezin *et al.*, 1990; Publicover *et al.*, 1993; Sanders, 1996; Ward *et al.*, 2000a). Therefore, ICC play an important role in the determination and regulation of GI motility. It has been found that ICC expressed the muscarinic, adrenergic, tachykinin, somatostatin and purinergic receptors using immunohistochemical or patch-clamp methods, suggesting that ICC are targets of a variety of endogenous substances (Vannucchi, 1999; Burnstock & Lavin, 2002; Kim *et al.*, 2003; Jun *et al.*, 2004a, b).

The effect of bradykinin on pacemaker activity, receptor subtypes and signal transduction pathways in ICC remains to be defined. Therefore, experiments were performed to determine whether bradykinin modulates pacemaker activities, to identify its receptor type and to determine specific signal transduction mechanisms in cultured ICC from murine small intestine. In addition, immunohistochemical staining was performed to localize bradykinin receptors in cultured ICC.

Methods

Preparation of cells and tissues

Balb/C mice (8–13 days old) of either sex were anesthetized with ether and killed by cervical dislocation. The small intestines from 1 cm below the pyloric ring to the cecum were removed and opened along the mesenteric border. Luminal contents were washed with Krebs–Ringer bicarbonate solution. The tissues were pinned to the base of Sylgard dish and the mucosa was removed by sharp dissection. Small stripes of intestinal muscle were equilibrated in Ca^{2+} -free Hank's solution (containing in mM: KCl 5.36, NaCl 125, NaOH 0.336, Na_2HCO_3 0.44, glucose 10, sucrose 2.9 and HEPES 11, adjusted to pH 7.4 with tris) for 30 min, and cells were dispersed with an enzyme solution containing collagenase (Worthington Biochemical Co., Lakewood, NJ, U.S.A.), 1.3 mg ml^{-1} ; bovine serum albumin (Sigma Chemical Co., St Louis, MO, U.S.A.), 2 mg ml^{-1} ; trypsin inhibitor (Sigma), 2 mg ml^{-1} and ATP, 0.27 mg ml^{-1} . Cells were plated onto sterile glass coverslips coated with murine collagen ($2.5 \mu\text{g ml}^{-1}$, 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, U.S.A.) supplemented with 2% antibiotics/antimycotics (Gibco, Grand Island, NY, U.S.A.) and murine stem cell factor (5 ng ml^{-1} , Sigma).

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. Recordings were made from cells within networks that had morphologies similar to the cells that were immunopositive for c-kit. Because the spontaneous inward currents from small groups of cells are more robust and regular than from single cells (Koh *et al.*, 1998), we recorded from small clusters of ICC. Currents or potentials were amplified by Axopatch 1-D or Axopatch 200B (Axon

Instruments, Foster, CA, U.S.A.). Command pulse was applied using an IBM-compatible personal computer and pClamp software (version 6.1 or 9.0; Axon Instruments). The data were filtered at 5 kHz and displayed on an oscilloscope, a computer monitor and a pen recorder (Gould 2200, Gould, Vally view, OH, U.S.A.).

Results were analyzed using pClamp and Graph Pad Prism (version 2.01) software. All experiments were performed at 30°C .

Solutions and drugs

The cells were bathed in a solution containing (mM): KCl 5, NaCl 135, CaCl_2 2, glucose 10, MgCl_2 1.2 and HEPES 10, adjusted to pH 7.4 with tris. The pipette solution contained (mM) KCl 140, MgCl_2 5, K_2ATP 2.7, Na_2GTP 0.1, creatine phosphate disodium 2.5, HEPES 5, EGTA 0.1, adjusted to pH 7.2 with tris.

Drugs used were as follows: bradykinin, bradykinin fragments 1–8 (selective bradykinin B1 receptor agonist), calphostin C, 4,4'-diisothocyanatostilbene-2,2'-disulfonic acid (DIDS), chelerythrine, naproxen, Lys-(des-Arg⁹,Leu⁸)-bradykinin tri-fluoroacetate salt (bradykinin B1 receptor antagonist), D-Arg-[Hyp³,Thi^{5,8},D-Phe⁷]-bradykinin (bradykinin B2 receptor antagonist) and thapsigargin. All drugs were purchased from the Sigma Chemical Co. [Hyp³,Tyr(Me)⁸]-bradykinin (bradykinin B2 receptor agonist) was purchased from the Calbiochem (La Jolla, CA, U.S.A.).

Statistical analysis

Data were expressed as means \pm s.e. Differences in the data were evaluated by Student's *t*-test. A *P*-value <0.05 was accepted as a statistically significant difference. The *n*-values reported in the text refer to the number of cells used in patch-clamp experiments.

Immunohistochemistry

Cultured ICC of the small intestine were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 10–20 min at 4°C . After fixation, cells were preincubated in phosphate-buffered saline containing 0.3% Triton X-100 and 1% bovine serum albumin for 1 h at room temperature. For double immunostaining, cells were incubated for 48 h at 4°C in a mixture of two primary antibodies raised in different species (anti-bradykinin B1 receptors: raised in goat, 1:200, Santa Cruz Biotechnology, CA, U.S.A.; anti-bradykinin B2 receptors: raised in goat, 1:200, Santa Cruz Biotechnology, CA, U.S.A.; anti-c-kit: raised in rat, 1:200, Gibco). The secondary antibodies were fluorescein isothiocyanate (FITC) for anti-bradykinin B2 receptors and Texas Red for c-kit, diluted 1:100 (all reagents from Vector Laboratories, Burlingame, CA, U.S.A.). The incubation of labeled secondary antibodies was performed for 1 h at room temperature. Control cells were prepared by omitting primary antibodies from the incubation solutions. Cells were examined with an FV300 confocal microscope (Olympus, Japan) with an excitation wavelength appropriate for FITC (488 nm) and Texas Red (568 nm). Final images were constructed with Flow View Software Program.

Results

Bradykinin effects on pacemaker activity in cultured ICC

Under a current clamp ($I=0$), ICC generated pacemaker potentials. The resting membrane potential was -59 ± 5 mV and amplitude was 28 ± 2 mV. Bradykinin (10 nM) produced membrane depolarization (Figure 1a). In the presence of bradykinin, membrane potentials were depolarized to -36 ± 5.4 mV and the amplitude of pacemaker potentials

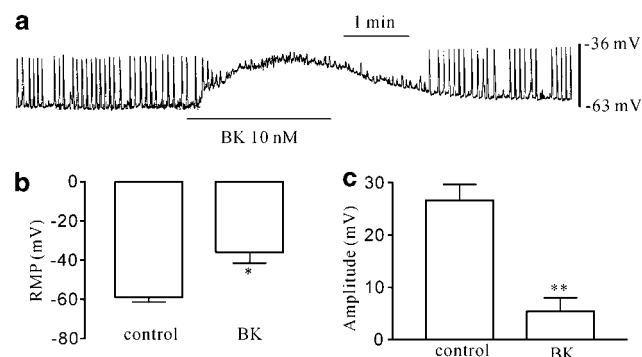


Figure 1 Effects of bradykinin on pacemaker potentials in cultured ICC of the murine small intestine. (a) Pacemaker potentials from ICC exposed to bradykinin (10 nM) in the current-clamp mode ($I=0$). Bradykinin caused membrane depolarization and decreased amplitude of pacemaker potentials. Responses to bradykinin are summarized in (b) and (c). Bars represent mean values \pm s.e. * $P < 0.05$, ** $P < 0.01$: significantly different from the untreated control.

was decreased to 5.4 ± 2.6 mV ($n=5$; Figure 1b and c). In addition, under a voltage clamp at a holding potential of -70 mV, bradykinin produced tonic inward currents and decreased the frequency and amplitude of pacemaker currents in a concentration-dependent manner (Figure 2a–d). The summarized values and bar graph of bradykinin effects on pacemaker currents are shown in Table 1 and Figure 2e–g ($n=6$). These results suggest that bradykinin modulates pacemaker activity in a dose-dependent manner in ICC.

Identification of receptor subtypes of bradykinin in cultured ICC

To identify the receptor subtypes of bradykinin, bradykinin receptor antagonists and agonists were used. Bradykinin B1 receptor (100 nM) or B2 receptor antagonist (100 nM) itself had no effect on pacemaker currents. In the presence of a bradykinin B1 receptor antagonist, bradykinin-induced responses on pacemaker currents were still present ($n=7$; Figure 3b). However, bradykinin B2 receptor antagonist almost completely blocked the bradykinin-induced responses to pacemaker currents ($n=8$; Figure 3c). Under controlled conditions at a holding potential of -70 mV, the resting currents were -24 ± 15 pA, and the frequency and the amplitude of pacemaker currents were 17 ± 0.5 cycles min^{-1} and -420 ± 32 pA, respectively. In the presence of the bradykinin B1 antagonist, the resting currents produced by bradykinin (100 nM) were -536 ± 41 pA, and the frequency and amplitude of pacemaker currents were 7 ± 0.9 cycles min^{-1} and -58 ± 12 pA, respectively. In the presence of bradykinin

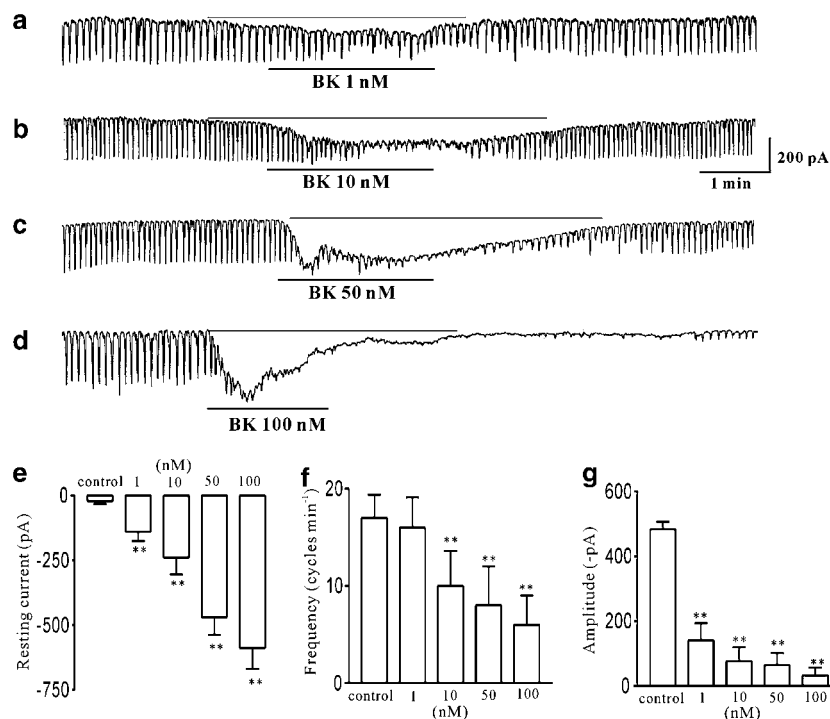


Figure 2 Effects of bradykinin on pacemaker currents in cultured ICC of murine small intestine. (a–d) Pacemaker currents from ICC exposed to bradykinin (1–100 nM) at a holding potential of -70 mV. Bradykinin caused a concentration-dependent increase in tonic inward currents and decrease in the frequency and amplitude of pacemaker currents. Responses to bradykinin are summarized in (e)–(g). Bars represent mean values \pm s.e. ** $P < 0.01$: significantly different from the untreated control. The dotted lines indicate zero current levels.

B2 antagonist, the resting currents produced by bradykinin (100 nM) were -58 ± 11 pA, and the frequency and amplitude of pacemaker currents were 16 ± 1.2 cycles min^{-1} and -410 ± 43 pA, respectively. The values of the bradykinin B1 antagonist were significantly different from those obtained with the bradykinin B2 antagonist (Figure 3e–g). To verify these results, we also used the selective B1 and B2 receptor agonists. Under controlled conditions at a holding potential of -70 mV, the selective B1 receptor agonist did not show any influence on pacemaker currents in ICC (Figure 3c). However, the selective B2 receptor agonist increased the tonic inward currents and reduced the frequency and amplitude of pacemaker currents (Figure 3d), and the B2 receptor agonist had the actions on ICC with EC_{50} values of 87 nM, respectively (graph not shown).

Table 1 Summarized values of bradykinin effects on pacemaker currents in ICC

		Resting currents (pA)	Frequency (cycles min^{-1})	Amplitude (pA)
Control		-21.7 ± 11	17 ± 0.4	483 ± 23
BK (nM)	1	$-140 \pm 36^{**}$	16 ± 3	$140 \pm 53^{**}$
	10	$-240 \pm 65^{**}$	$10 \pm 3.6^{**}$	$76 \pm 53^{**}$
	50	$-470 \pm 68^{**}$	$8 \pm 4^{**}$	$64 \pm 37^{**}$
	100	$-589 \pm 80^{**}$	$6 \pm 3^{**}$	$32 \pm 24^{**}$

Abbreviations: BK, bradykinin.

$^{**}P < 0.01$: significantly different from the untreated control ($n = 6$).

Effects of the external Na^+ -free or Cl^- channel blocker in bradykinin-induced responses in cultured ICC

To determine the characteristics of the tonic inward currents produced by bradykinin, an external Na^+ -free solution and a Cl^- channel blocker were tested. External Na^+ was substituted for by the same concentrations of *N*-methyl-D-glucamine. In the presence of an external Na^+ -free solution, pacemaker current was abolished. Under these conditions, bradykinin (100 nM) did not produce tonic inward currents ($n = 8$; Figure 4a). For the external Na^+ -free solution, the resting currents produced by bradykinin were -27 ± 9 pA, this value is significantly different when compared to the normal control solution (Figure 4c). In the presence of the application of DIDS (10 μM), a Cl^- channel blocker, the pacemaker currents were abolished. In these conditions, bradykinin still produced tonic inward currents ($n = 7$; Figure 4b). In the pretreatment of DIDS, the resting currents produced by bradykinin were -469 ± 19 pA, this value was not significantly different when compared with control values obtained in the absence of DIDS (Figure 4d).

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

To investigate the role of external Ca^{2+} or internal Ca^{2+} , bradykinin was tested under external Ca^{2+} -free conditions and in the presence of thapsigargin, a Ca^{2+} -ATPase inhibitor of endoplasmic reticulum. Pacemaker currents recorded at a holding potential of -70 mV were completely abolished by external Ca^{2+} -free solution. In this condition, bradykinin-

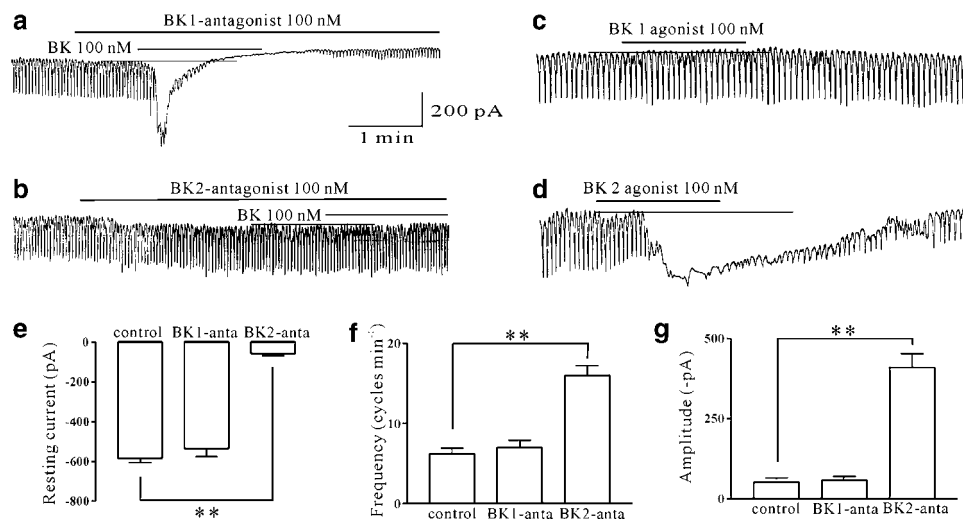


Figure 3 Effects of bradykinin antagonists on bradykinin-induced responses or selective bradykinin B1 and B2 receptor agonists on pacemaker currents in cultured ICC of the murine small intestine. (a) Pacemaker currents of ICC exposed to bradykinin (100 nM) in the presence of bradykinin B1 receptor antagonist (100 nM). In the presence of bradykinin B1 receptor antagonist, bradykinin still caused tonic inward currents and decrease in the frequency and amplitude of pacemaker currents. (b) Pacemaker currents of ICC exposed to bradykinin (100 nM) in the presence of bradykinin B2 receptor antagonist (100 nM). Bradykinin B2 receptor antagonists blocked the effects of bradykinin on pacemaker currents. (c) Pacemaker currents of ICC exposed to the selective bradykinin B1 receptor agonist (100 nM). The selective bradykinin B1 receptor agonist did not have any influence on pacemaker currents. (d) Pacemaker currents of ICC exposed to the selective bradykinin B2 receptor agonist (100 nM). The selective bradykinin B2 receptor agonist caused tonic inward currents and decrease in the frequency and amplitude of pacemaker currents. Responses to bradykinin are summarized in (e)–(g). Bars represent mean values \pm s.e. $^{**}P < 0.01$: Significantly different from the untreated control. The dotted lines indicate the zero current levels.

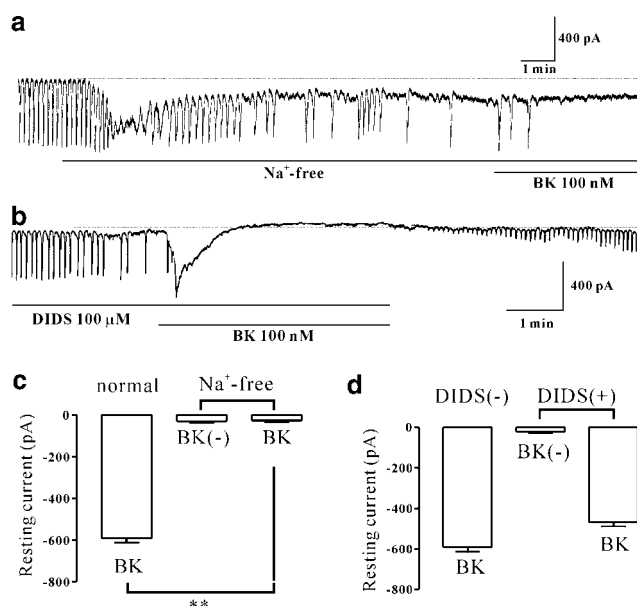


Figure 4 Effects of an external Na^+ -free medium or DIDS, a Cl^- channel blocker, on bradykinin-induced responses on pacemaker currents in cultured ICC of the murine small intestine. (a) The external Na^+ -free solution abolished the generation of pacemaker currents. Under these conditions, bradykinin did not produce tonic inward currents. (b) Also, DIDS ($10 \mu\text{M}$) abolished the generation of pacemaker currents. However, DIDS did not block the bradykinin-induced tonic inward currents. Responses to bradykinin in the external Na^+ -free solution and in the presence of DIDS are summarized in (c) and (d). Bars represent mean values \pm s.e. $^{**}P < 0.01$: significantly different from the untreated control. The dotted lines indicate the zero current levels.

induced tonic currents were suppressed ($n = 8$; Figure 5a). In the external Ca^{2+} -free solution, the tonic inward currents produced by bradykinin were $-140 \pm 48 \text{ pA}$. These values were significantly different when compared with bradykinin in normal Ca^{2+} solution (Figure 5d). In addition, bradykinin-induced tonic inward currents were suppressed with pretreatment use of thapsigargin (Figure 5b). In the presence of thapsigargin ($5 \mu\text{M}$), the tonic inward currents produced by bradykinin were $-52 \pm 14 \text{ pA}$. These values were significantly different when compared to bradykinin in the absence of thapsigargin ($n = 5$; Figure 5e). Also, to investigate whether the voltage-dependent Ca^{2+} channels are involved in the actions of bradykinin on pacemaker currents in ICC, we used nifedipine, a voltage-dependent Ca^{2+} channel blocker. Nifedipine ($1 \mu\text{M}$) had no effect on pacemaker currents, and in this condition, bradykinin still increased tonic inward currents and reduced the frequency and amplitude of pacemaker currents (Figure 5b).

Effects of protein kinase C inhibitor or cyclooxygenase inhibitor in bradykinin-induced responses in cultured ICC

We tested the effects of chelerythrine and calphostin C or naproxen to investigate whether bradykinin-induced responses to pacemaker currents are mediated by the activation of protein kinase C or the production of prostaglandins. Chelerythrine ($1 \mu\text{M}$), calphostin C ($1 \mu\text{M}$) and naproxen ($10 \mu\text{M}$) did not have an effect on pacemaker currents and is summarized in Figure 6d, the value also was not significantly different when compared with control values obtained in the

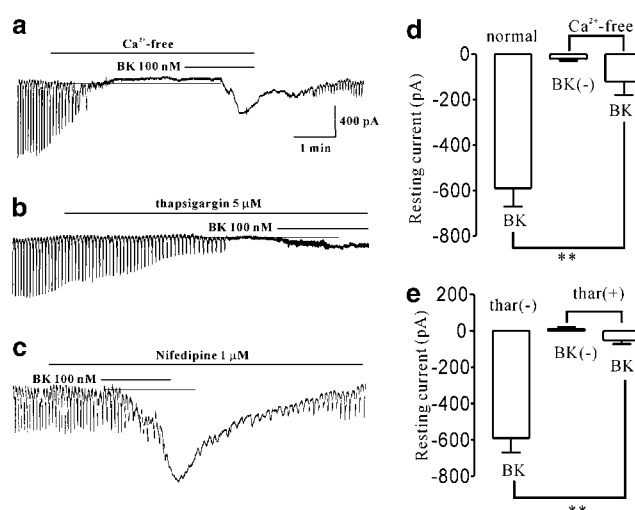


Figure 5 Effects of an external Ca^{2+} -free solution, thapsigargin or nifedipine upon bradykinin-induced responses on pacemaker currents in cultured ICC of the murine small intestine. (a) The external Ca^{2+} -free solution abolished the generation of pacemaker currents. Under this condition, bradykinin-induced tonic inward currents were suppressed. (b) Thapsigargin ($5 \mu\text{M}$) abolished the generation of pacemaker currents. However, thapsigargin blocked the bradykinin-induced tonic inward currents. Responses to bradykinin in the external Ca^{2+} -free solution and in the presence of thapsigargin are summarized in (d) and (e). (c) The treatment of nifedipine ($1 \mu\text{M}$) on ICC did not show any effect on pacemaker currents, and also in this statement, bradykinin (100 nM) still showed tonic inward currents. Bars represent mean values \pm s.e. $^{**}P < 0.01$: significantly different from the untreated control. The dotted lines indicate the zero current levels.

absence of chelerythrine, calphostin C and naproxen ($n = 7$). Pretreatment with chelerythrine, calphostin C and naproxen did not block the bradykinin-induced responses of pacemaker currents (Figure 6a–c).

Immunohistochemistry for bradykinin B2 receptors in cultured ICC

Expression of bradykinin B1 or bradykinin B2 receptors were investigated by immunohistochemistry on cultured ICC. Double staining with anti-c-kit (a marker of ICC) and anti-bradykinin B2 receptor antibodies revealed a colocalization of bradykinin B2 receptor- and c-kit-like immunoreactivities in cultured ICC (Figure 7d–f). However, double staining with anti-c-kit (a marker of ICC) and anti-bradykinin B1 receptor antibodies revealed only a localization of c-kit-like immunoreactivity in cultured ICC (Figure 7a–c).

Discussion

In this study, we found that bradykinin depolarized the membrane potential by producing tonic inward pacemaker currents of ICC in the murine small intestine. This process is mediated by the release of 1,4,5-inositol triphosphate (IP_3)-dependent intracellular Ca^{2+} release from endoplasmic reticulum and by external Ca^{2+} influx through bradykinin B2 receptor activation.

Bradykinin stimulates contraction or relaxation of GI smooth muscles depending on species and tissues. Bradykinin

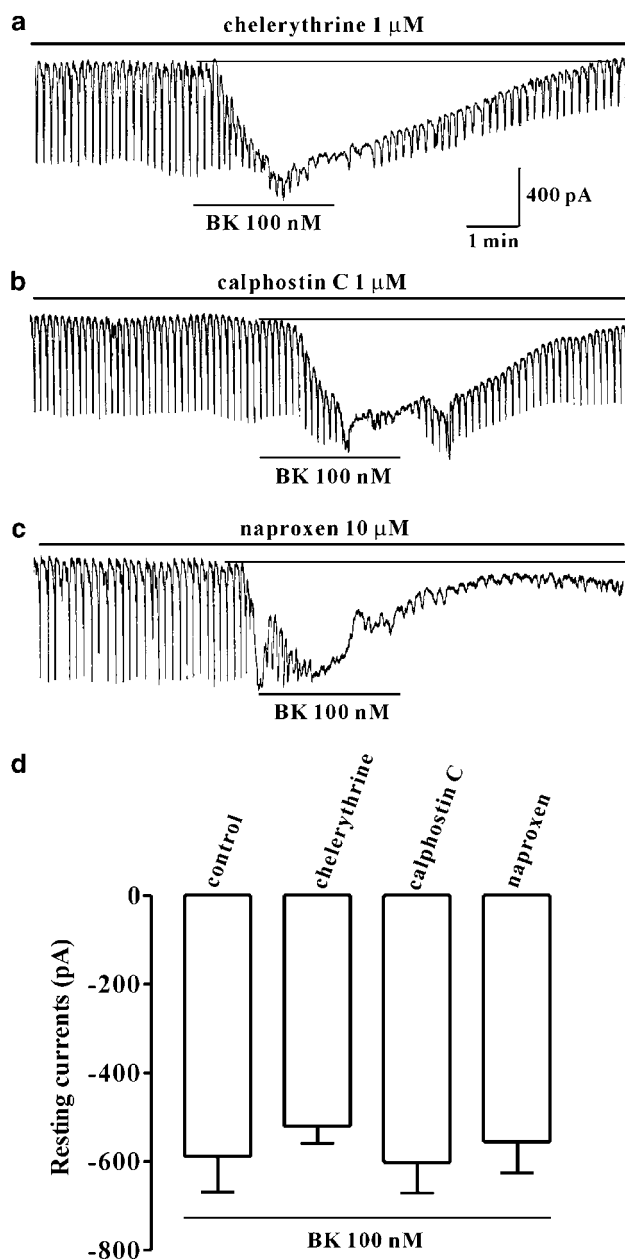


Figure 6 Effects of chelerythrine, calphostin C (protein kinase C inhibitors) or naproxen (a cyclooxygenase inhibitor) upon bradykinin-induced responses on pacemaker currents in cultured ICC of the murine small intestine. (a, b) Pacemaker currents of ICC exposed to bradykinin (100 nM) in the presence of chelerythrine (1 μ M) or calphostin C (1 μ M). In this condition, bradykinin caused tonic inward currents and decreased the frequency and amplitude of pacemaker currents. (c) Pacemaker currents of ICC exposed to bradykinin (100 nM) in the presence of naproxen (10 μ M). Naproxen did not block the effects of bradykinin on pacemaker currents. Responses to bradykinin 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.

contracts colonic, ileal and duodenal smooth muscles (Feres *et al.*, 1992; Calixto, 1995; Hasler *et al.*, 1995). In contrast, bradykinin relaxes human colonic smooth muscles and rat duodenal smooth muscles (Boschcov *et al.*, 1984; Wassdal *et al.*, 1999). The action of bradykinin in GI smooth muscles is mediated by the activation of G-protein-coupled cell membrane receptors. The GI smooth muscles express two subtypes

of bradykinin receptors, B1 and B2 (Hall, 1997). Bradykinin B2 receptors are constitutively expressed in normal tissue and have a wide distribution and mediate the actions of bradykinin under normal conditions and during acute inflammation. However, the B1 receptors are expressed at low levels under normal conditions, but expression is upregulated following tissue damage or inflammation (Farmer & Burch, 1992; Hall, 1997). Generally, bradykinin B2 receptor activation mediates the contractions of GI smooth muscles (Regoli, 1983). In the present study, we tested the effects of bradykinin on pacemaker currents in the presence of Lys-(des-Arg⁹,Leu⁸)-bradykinin trifluoroacetate salt (bradykinin B1 receptor antagonist) and D-Arg-[Hyp³,Thi^{5,8},D-Phe⁷]-bradykinin (bradykinin B2 receptor antagonist) that are reported to strongly antagonize the action of bradykinin than other B1 or B2 bradykinin receptor antagonists (Schachter *et al.*, 1987; MacNeil *et al.*, 1997). First, we analyzed the effect of 100 nM BK on pacemaker currents in the presence of various concentrations of BK2 receptor antagonist and we found that 100 nM BK2 antagonist completely blocked the tonic inward current induced by 100 nM BK. Also, we found that the BK2 antagonist competitively inhibited the action of BK (data not shown). However, bradykinin B1 receptor antagonist did not block the bradykinin-induced responses on pacemaker activity. To verify this result, we tested the effects of bradykinin fragments 1–8 (selective bradykinin B1 receptor agonist) and [Hyp³,Tyr(Me)⁸]-bradykinin (bradykinin B2 receptor agonist) on pacemaker currents. It was reported that the bradykinin B1 receptor has a high affinity for bradykinin fragments 1–8 (Naraba *et al.*, 1998) and [Hyp³,Tyr(Me)⁸]-bradykinin specifically binds the bradykinin B2 receptors (Galizzi *et al.*, 1994). In our results, only the bradykinin B2 receptor agonist showed the effects on pacemaker currents with EC₅₀ value of 87 nM. In addition, ICC revealed a colocalization of bradykinin B2 receptor and c-kit immunoreactivities. These findings suggest that effects of bradykinin are mediated by the BK2 receptor in ICC.

ICC generate spontaneous pacemaker currents that depolarize membrane potentials, which spread to smooth muscle *via* gap junctions resulting in depolarization of the membrane in smooth muscle. The membrane depolarization leads to contraction in smooth muscle by generating action potentials through activation of voltage-dependent Ca²⁺ channels. It has been suggested that pacemaker currents of ICC are mediated by the activation of voltage-independent nonselective cation channels (Koh *et al.*, 1998; Kim *et al.*, 2005) or Cl[−] channels (Huizinga *et al.*, 2002). The activation of nonselective cation channels allows net inward current carried predominantly by Na⁺ under physiological conditions; this leads to excitatory action in GI smooth muscles (Kuriyama *et al.*, 1998). In the present study, the external Na⁺-free solution abolished the generation of pacemaker currents. This result suggests that nonselective cation channels are involved in generating pacemaker currents in ICC. In the present study, the bradykinin-induced tonic inward current was suppressed by external Na⁺-free solution. However, DIDS, a Cl[−] channel blocker, did not block the bradykinin-induced tonic inward currents, suggesting that bradykinin may regulate nonselective cation channels. These findings are similar to the effects shown in submucosal enteric neurons by bradykinin. Bradykinin depolarizes membrane potentials of submucosal enteric neurons and the amplitude of depolarizing responses by bradykinin is reduced in low Na⁺ solution, suggesting that bradykinin activates

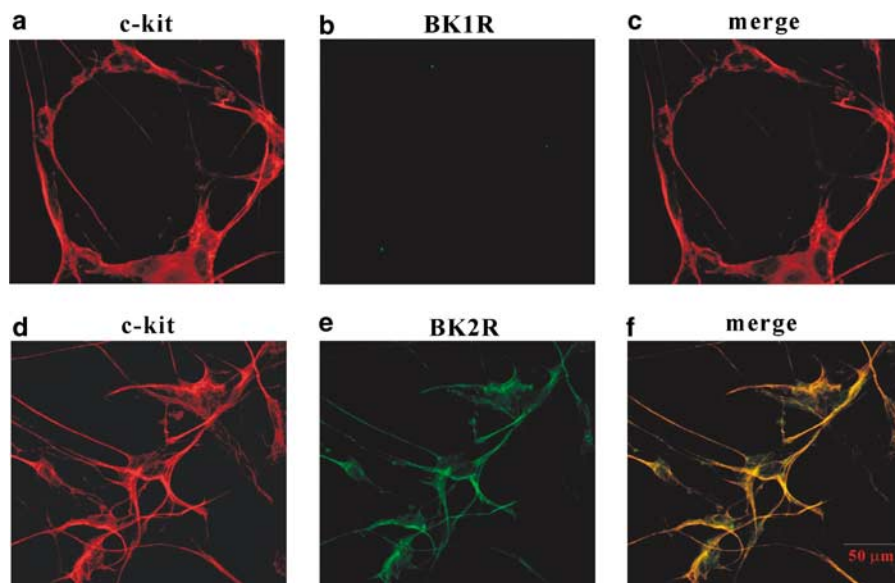


Figure 7 Expression of the bradykinin B2 receptor was shown by immunohistochemistry on cultured ICC. (a) Kit-immunopositive ICC network. (b) Bradykinin B1 receptor-immunonegative ICC network. (c) Double staining with anti-bradykinin B2 receptors and anti-c-kit antibodies revealed only a localization of c-kit immunopositive ICC. (d) Kit-immunopositive ICC network. (e) Bradykinin B2 receptor-immunopositive ICC network. (f) Double staining with anti-bradykinin B2 receptors and anti-c-kit antibodies revealed a colocalization (merge) in cultured ICC.

a cation conductance in the submucosal enteric neuron (Hu *et al.*, 2004). In addition, we previously reported that substance P can also activate nonselective cation channels in cultured ICC of small intestine (Jun *et al.*, 2004b).

The external Ca^{2+} influx is necessary for GI smooth muscle contractions. It has been reported that acetylcholine and substance P activated the nonselective cation channels leading to depolarization and opening of voltage-dependent Ca^{2+} channels, and thereby cause contraction (Kuriyama *et al.*, 1998). Also, bradykinin-mediated contractions in guinea-pig ileum and urinary bladder, and rat vas deferens were markedly attenuated in Ca^{2+} -free medium (Calixto, 1995). In addition, external Ca^{2+} was essential to generate pacemaker currents in ICC. In Ca^{2+} -depleted medium, the pacemaker currents were abolished (Tokutomi *et al.*, 1995). In the present study, bradykinin-induced tonic inward current was suppressed in the external Ca^{2+} -free solution. This means that external Ca^{2+} influx is partially necessary for bradykinin-induced tonic inward pacemaker currents. Bradykinin-mediated contractions in guinea-pig ileum and urinary bladder, and rat vas deferens are sensitive to nicardipine, suggesting that bradykinin activates L-type voltage-dependent Ca^{2+} channels (Calixto, 1995). However, in this study, nifedipine did not block the bradykinin-induced tonic inward currents in ICC, indicating that L-type voltage-dependent Ca^{2+} channels are not involved here. This discrepancy between smooth muscle and ICC may be owing to regional differences. Possible regional differences may explain why the action of bradykinin on guinea-pig gallbladder and ileal smooth muscles was not blocked by nicardipine (Calixto & Medeiros, 1991; Cabrini *et al.*, 1995).

Bradykinin B2 receptors activate phospholipase C, which in turn increases IP_3 and diacylglycerol. IP_3 mobilizes Ca^{2+} from endoplasmic reticulum and diacylglycerol activates protein kinase C (Ellis & Fozard, 2002). Bradykinin contracts colon circular muscles or ileal longitudinal muscles by IP_3 generation and elevates cytosolic Ca^{2+} concentration in rat duodenal

smooth muscle cells by the release of Ca^{2+} from internal storage (Ransom *et al.*, 1992; Hasler *et al.*, 1995; Wassdal *et al.*, 1999). In addition, the generation of pacemaker currents was dependent on intracellular Ca^{2+} oscillation. The periodic release of Ca^{2+} from endoplasmic reticulum is essential for generating pacemaker currents (Ward *et al.*, 2000b). In the present study, thapsigargin, a potent endoplasmic reticulum Ca^{2+} -ATPase inhibitor, suppressed the bradykinin-induced tonic inward currents. These results suggest that the release of Ca^{2+} from internal storage by bradykinin is essential to activate tonic inward currents. In isolated guinea-pig ileal smooth muscle, bradykinin-induced contractions are partially attenuated by H-7, a protein kinase C inhibitor, suggesting that protein kinase C is involved in bradykinin action (Calixto & Medeiros, 1991). In addition, the protein kinase C activator also initiates tonic inward currents in cultured gastric ICC (Kim *et al.*, 2003). However, in the present study, chelerythrine or calphostin C, specific and potent protein kinase C inhibitors, did not block these bradykinin-induced effects, suggesting that protein kinase C is not involved in bradykinin B2 receptor stimulation.

Several papers have reported the involvement of cyclooxygenase and prostaglandins in bradykinin-induced effects (Leduc *et al.*, 1994; Ellis & Fozard, 2002; Hu *et al.*, 2003). Prostaglandins are generated through the activity of cyclooxygenase and play a significant role in its physiology in the GI tract (Whittle & Vane, 1987). In the present study, we found that the bradykinin-induced tonic current was not blocked by naproxen, a cyclooxygenase inhibitor, indicating that the production of prostaglandins is not involved in stimulating bradykinin effects.

In conclusion, this study describes the effects of bradykinin B2 receptors on ICC in the murine small intestine. Bradykinin depolarized the membrane with increased tonic inward currents, which was activated by nonselective cation channels *via* external Ca^{2+} influx and Ca^{2+} release from internal

storage in a protein kinase C- or cyclooxygenase-independent manner. Thus, the bradykinin B2 receptor may play an important role in the regulation of GI motility by acting on ICC.

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