Calcitonin gene-related peptide inhibits pacemaker activities by the activation of ATP-sensitive K^+ channels via G-protein pathway in cultured interstitial cells of Cajal from mouse small intestine

배양된 생쥐 소장의 interstitial cells of Cajal 에서 calcitonin gene-related peptide 의 향도잡이 활동도에 대한 억제기전

2005 年 2 月 日

朝鮮大學校 大學院

醫 學 科

車 京 勳

Calcitonin gene-related peptide inhibits pacemaker activities by the activation of ATP-sensitive K^+ channels via G-protein pathway in cultured interstitial cells of Cajal from mouse small intestine

指導敎授　　朴 英 奉

이 論文을 醫學博士學位 申請論文으로 제출함

2004 년　10　월　　일

朝鮮大學校 大學院

醫 學 科

車　京　勳

車京勳의 博士學位論文을 認准함

委員長	조선大學校 敎授	박 상 기	印
委 員	조선大學校 敎授	문 경 래	印
委 員	조선大學校 敎授	염 철 호	印
委 員	조선大學校 敎授	전 제 열	印
委 員	조선大學校 敎授	박 영 봉	印

2004 年 12 月 日

朝 鮮 大 學 校 大 學 院

목 차

Legends for Figures

Fig. 1. Kit-immunoreactive ICC in cell culture.

Fig. 2. The typical traces of pacemaker potential and pacemaker currents recorded in cultured ICC of murine small intestine.

Fig. 3. Effects of CGRP on pacemaker potentials in cultured ICC of murine small intestine.

Fig. 4. Effects of CGRP on pacemaker currents in cultured ICC of murine small intestine.

Fig. 5. Effects of pinacidil on pacemaker potentials and pacemaker currents in cultured ICC of murine small intestine.

Fig. 6. Effects of 5-hydroxydecanoic acid on pinacidil-induced effects in cultured ICC of murine small intestine.

Fig. 7. Effects of GDP β S on the response to CGRP in cultured ICC of murine small intestine.

Fig. 8. Effects of adenylate cyclase inhibitor on the response to CGRP in cultured ICC of murine small intestine.

Fig. 9. Effects of guanylate cyclase inhibitor on the response to CGRP in cultured ICC of murine small intestine.

Fig. 10. Effects of cyclooxygenase or nitric oxide synthase inhibitor on the response to CGRP in cultured ICC of murine small intestine.

국 문 초 록

배양된 생쥐 소장의 Cajal세포에서 CGRP에 의한 향도잡이 전류의 억제기전

차경훈

지도교수 : 박영봉

조선대학교 의과대학원 의학과

위장관 Cajal세포는 자발적 수축을 야기하는 서파의 근간인 자발적 내향성 전류, 즉 향도잡이 전류를 발생시킨다. 또한 Cajal세포는 내인성 장관 신경계로부터 신호를 전달 받아 평활근으로 매개하는 역할을 통하여 위장관 운동성을 조절하고 있다. CGRP(calcitonin gene-related peptide)는 위장관 신경계로부터 유리되는 펩타이드 물질로서 일반적으로 위장관 운동성을 억제하는 것으로 알려져 있다. 본 연구는 CGRP의 위장관 운동성 조절 억제가 Cajal세포를 매개로 이루어지는 가를 알아보고자 생쥐 소장에서 배양된 Cajal세포에서 세포막 전압 고정법을 사용하여 이 전류에 대한 CGRP의 효과와 작용을 규명하고자 하였다. CGRP를 투여한 경우 향도잡이 전류의 크기가 감소되고 발생 빈도가 억제되었으며 외향성 전류가 발생되었다. 또한 세포막의 과분극이 초래되었다. 전류와 전압에 대한 CGRP의 효과는 ATP-민감성 K^+ 통로 차단제인 glibenclamide에 의해서

차단되었다. ATP-민감성 K^+ 통로 개방제인 pinacidil 또한 CGRP와 동일한 효과를 나타냈으며 glibenclamide에 의해서 차단되었다. Adenylate cyclase 억제제인 SQ-22536, guanylate cyclase 억제제인 ODQ, NOS 억제제인 L-NAME 및 cyclooxygenase 억제제인 naproxen는 향도잡이 전류에 대한 CGRP의 작용을 차단하지 못했다. 그런 반면 G-단백질을 억제하는 GDPβS에 의해서 CGRP의 작용이 크게 억제 되었다. 이상의 실험 결과는 CGRP가 G-단백질을 매개하여 ATP-민감성 K^+ 통로를 활성화 시켜 Cajal세포에서 발생되는 향도잡이 전류를 억제함을 나타내며 이는 cyclic AMP, cyclic GMP, NO 및 prostaglandins생성과는 무관하게 이루어지는 것으로 사료된다.

중심단어 : Cajal 세포, 향도잡이 전류, ATP-민감성 K^+ 통로, CGRP, 소장

1. Introduction

CGRP (Calcitonin gene-related peptide) is a neuropeptide that distributed in the central and peripheral nervous systems including enteric nervous system in the gastrointestinal tract. CGRP is identified immunohistochemistry in both myenteric and submucosal plexus and supply all layers of the intestinal wall along the entire gastrointestinal tract [1]. The distribution of CGRP in the gastrointestinal tract strongly suggests an important role for CGRP in function. Indeed, CGRP involves in the regulation of gastric acid secretion, gastric and mesenteric blood flow, and modulation of gastrointestinal motility [2]. Generally, the effect of CGRP on gastrointestinal motility is predominantly relaxation by acting on smooth muscle cells directly [3, 4]. However, the indirect contracting effects have been reported by the release of acetylcholine from enteric neurons [5]. Also, there is increasing evidence that under pathological conditions CGRP is released from enteric nerve fibers in the gut and cause cessation of propulsive motor activity [6]. The gastrointestinal smooth muscles show spontaneous mechanical contractions, which are initiated by periodic membrane depolarizations, called slow waves. Slow waves determine the frequency and timing of smooth muscle contraction [7].

Interstitial cells of Cajal (ICC) are pacemaker cells that regulate gastrointestinal motility by generating electrical slow waves [8-9]. They form electrically coupled network between the circular and longitudinal muscle layer, between bundles of smooth muscle cells, and along the submucosal surface of the circular muscle layer in gastrointestinal tract. ICC are coupled to neighboring smooth muscle cells through low resistance electrical gap junctions. Thus slow waves of ICC are spread to smooth

muscles [10]. Also, ICC propagate slow waves through a voltage-dependent Ca^{2+} conductance mechanism [11]. In addition, ICC are closely associated with varicosities of the enteric nerve, which mediate inhibitory and excitatory nerve signals to smooth muscle [12, 13]. Therefore, these cells play an important role as basic regulators of gastrointestinal motility. Moreover, disruption to these cells, such as, a reduction in their numbers or changes to their morphological patterns, produce pathological motility disorders in the gastrointestinal tract [10, 14]. Hormones and enteric neurotransmitters regulate the gastrointestinal mechanical contractions and electrical activities by modulating slow waves [15]. These relations suggest that the ICC are physiological and therapeutic targets for numerous hormones, neurotransmitters, and drugs. In the enteric nervous system numerous neurotransmitters are released and regulate gastrointestinal motility. Neurotransmission is mediated not only by the classical neurotransmitters but also by neuropeptide transmitters. It is well known that major excitatory neurotransmitters are acetylcholine and substance P, whereas the major inhibitory neurotransmitters are nitric oxide and vasoactive intestinal polypeptide (VIP) [16]. Recently, the modulatory effects of muscarinic, tachykinin, and adrenergic β-receptor stimulation on pacemaker currents in cultured ICC have been reported [17-19]. Besides somatostatin, VIP, and purinergic receptors are identified by immunohistochemistry [20, 21]. Therefore, CGRP also can regulate intestinal motility indirectly by acting on ICC. However, there are no any reports on the CGRP effect to the electrical activity in ICC. In this study, we investigated the effect of CGRP on the electrical activity of ICC and the its underlying mechanisms in cultured ICC from murine small intestine.

2. Methods

2.1. Preparation of cells

Balb/C mice (8-13 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. Luminal contents were washed away with Krebs-Ringer bicarbonate solution. The tissues were pinned to the base of Sylgard dish and the mucosa 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 for 30 min and cells were dispersed with an enzyme solution containing collagenase (Worthington Biochemical Co, Lakewood, NJ, USA), 1.3 mg/ml, bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA), 2 mg/ml, trypsin inhibitor (Sigma), 2 mg/ml and ATP, 0.27 mg/ml. Cells were plated onto sterile glass coverslips coated with murine collagen (2.5 μg/ml, Falcon/BD) in 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).

2.2 Labeling of cultured ICC by c-Kit immunofluorescence

Cultured ICC was fixed in acetone (20°C/5 min). Following fixation, preparations were

washed for 60 min in phosphate-buffered saline (PBS; 0.01 M, pH 7.4). Cultured ICC were then incubated in 10% goat serum containing 1% bovine serum albumin for 1 h at RT to reduce nonspecific antibody binding. To examine the ICC, cultured ICC were incubated overnight at 4°C with a rat monoclonal antibody raised against Kit protein (ACK_2; 5 μg ml^{-1} in PBS; Gibco-BRL, Gaithersburg, MD, U.S.A). Immunoreactivity was detected using fluorescein isothiocyanate (FITC)-conjugated secondary antibody (FITC-anti-rat; Vector Laboratories, 1:100 in PBS, 1h, room temperature). Control cultured ICC were prepared in a similar manner, but omitting ACK_2 from the incubation medium. Cells were examined under a confocal laser scanning microscope (FV300, Olympus, Japan) at an excitation wavelength appropriate for FITC (488nm) (Fig.1).

2.3. 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 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, Vally view, OF, USA).

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

2.4. 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 7.2 with tris. The pipette solution contained (mM): K-aspartate, 20; KCl, 120; $MgCl_2$, 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: Pinacidil, glibenclamide, calcitonin gene related peptide, 8-bromo-cyclic AMP, 8-bromo-cyclic GMP, SQ-22536, ODQ, 5-hydroxydecanoic acid (5-HD), diazoxide, GDP β S, L-NAME, naproxen. All drugs were purchased from the Sigma Chemical Co.

2.5. Statistical analysis

Data were expressed as means ± standard errors. 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 patch-clamp experiments.

3. Results

3.1. Spontaneous inward pacemaker currents in ICC

Under a current clamp, ICC generated pacemaker potentials. The resting membrane potential was –59 ± 5 mV and amplitude was 28 ± 2 mV (n=13; Fig. 2A). Under a voltage clamp at a holding potential of –70 mV, ICC generated spontaneous inward currents, which have been called 'pacemaker currents' [22, 23]. The frequency of the pacemaker current was 16 ± 2 cycles min^{-1} and the amplitude was –413 ± 61 pA (n=33; Fig. 2B).

3.2. Effects of CGRP on pacemaker activity

Under current clamp, CGRP (5 x 10^{-7} M) hyperpolarized the membrane potential from control value of –62 ± 1.4 mV to –69.8 ± 1.1 mV (n=12) (Fig. 3A and B). And the amplitude of pacemaker potential by CGRP was decreased to 2.9 ± 1.1 mV from a control value of 23 ± 2 mV. The CGRP-induced change of pacemaker potential was blocked by the application of glibenclamide, an inhibitor of ATP-sensitive K^+ channels. Glibenclamide (10^{-5} M), recovered to –61 ± 1.6 mV of resting membrane potential and to 21.7 ± 1.7 mV of amplitude of pacemaker potential (Fig. 3A and C). Under voltage clamp at a –70 mV of holding potential, ICC generated spontaneous inward pacemaker currents. CGRP increased resting currents to the outward and decreased frequency and amplitude of pacemaker currents (Fig. 4A). The increased outward current by CGRP was 136 ± 14.4 pA (Fig. 4B). Frequency was decreased on average from a control value

of 17 ± 0.5 cycles min^{-1} to 6.8 ± 1.9 cycles min^{-1} (Fig. 4C) and amplitude of pacemaker currents was decreased on average from a control value of –482.7 ± 22.2 pA to –45 ± 10.3 pA (Fig. 4D). Glibenclamide inhibited CGRP-induced effects on pacemaker currents. Glibenclamide recovered the resting currents to normal level, and frequency to 16.4 cycles min^{-1}, and amplitude of pacemaker currents to –482.4 ± 16.1 pA (Fig. 4A). Pinacidil, an ATP-sensitive K^+ channel opener, showed the mimicked effects with CGRP. Pinacidil (10^{-5} M) produced membrane hyperpolarization in current clamping mode and inhibited the amplitude and frequency of pacemaker currents and increased resting currents in the outward direction in voltage clamping mode (n=7) (Fig. 5A and B).

To rule out the mitochondrial ATP-sensitive K^+ channel in cultured ICC, we tested the 5-hydroxydecanoic acid (a selective mitochondrial ATP-sensitive K^+ channel blocker) or diazoxide (a mitochondrial ATP-sensitive K^+ channel opener). 5-hydroxydecanoic acid (5 x 10^{-4} M) did not block the pinacidil-mediated effects (n=5, Fig. 6A). Also, diazoxide (10^{-5} M) had no effect on inward pacemaker currents (n=4, Fig. 6B)

3.3 Involvement of G-protein in CGRP-mediated inhibition of pacemaker currents.

The effects of GDP β S, a nonhydrolysable guanosine 5'-diphosphate analogue, which permanently inactives GTP-binding proteins, were examined to determine whether the G-protein is involved in the effect of CGRP in ICC. The inhibitory effect of CGRP was blocked when GDP β S (1 mM) was in the pipette (Fig. 7A). Under control conditions at a holding potential of -70 mV, the frequency and amplitude were 16±4 cycles min-1

and -425 ± 37 pA, respectively (n=5). The addition of CGRP decreased and both the frequency and the amplitude of pacemaker currents (frequency: 2.7 ± 0.9 cycles min-1; amplitude: -20.8 ± 18 pA). In the presence of GDP β S in the pipette, the effects of CGRP on these variables were significantly attenuated; the frequency and amplitude were 16 ± 4 cycles min-1 and -348 ± 58 pA, respectively, These values are significantly different from those obtained with CGRP alone. This indicates that G-proteins have an essential role in CGRP-induced effects on pacemaker currents in ICC.

3.4. Effects of adenylate cyclase and guanylate cyclase inhibitor on the CGRP-induced effects on pacemaker currents.

The effects of SQ-22536, an inhibitor of adenylate cyclase, were examined to investigate possible regulation of pacemaker currents by the cyclic AMP-dependent pathway in CGRP-mediated inhibition. SQ-22536 was pretreated for 10 min before the application of CGRP. In the presence of SQ-22536 (10^{-5} M), CGRP still inhibited the pacemaker currents (n=5) (Fig. 8A). In the presence of SQ-22536, the resting currents was 120 ± 17.3 pA, and the frequency and amplitude of the pacemaker currents were 4.5 ± 2.1 cycles min^{-1} and –36 ± 18 pA, ($P<0.05$) respectively. In addition, cell permeable 8-bromo-cAMP (10^{-4} M) did not affect on the generation of pacemaker currents (n=8) (Fig. 8B). On the other hand 8-bromo-cGMP inhibited the pacemaker currents. Under control conditions at a holding potential of –70 mV, the resting currents was –32 ± 19 pA, and the frequency and the amplitude of the pacemaker currents were 17. 7 ± 0.5 cycles min^{-1} and –412 ± 29 pA, respectively. In the presence of 8-bromo-

cGMP (10^{-4} M), the frequencies and amplitudes decreased to 2.1 ± 0.5 cycles min^{-1} and –13 ± 16 pA (n=7, $P<0.05$) (Fig. 9A). However, ODQ, an inhibitor of guanylate cyclase, did not block the CGRP–mediated inhibition of pacemaker currents. ODQ was pretreated for 10 min before the application of CGRP. In the presence of ODQ (10^{-5} M) CGRP still inhibited the pacemaker currents (n=4) (Fig. 9B). In the presence of ODQ, the resting currents was 140 ± 21 pA, and the frequency and amplitude of the pacemaker currents were 3.4 ± 1.8 cycles min^{-1} and –27 ± 13 pA, ($P<0.05$) respectively.

3.5. Effects of naproxen or L-NAME on the CGRP-mediated inhibition of pacemaker currents.

The effects of naproxen (an inhibitor of cyclooxygenase) or L-NAME (an inhibitor of nitric oxide synthase) were examined to investigate possible regulation of pacemaker currents by prostaglandins or nitric oxide in CGRP-mediated inhibition. L-NAME or naproxen was pretreated for 10 min before the application of CGRP. In the presence of naproxen (5 x 10^{-6} M) or L-NAME (10^{-5} M), CGRP (5 x 10^{-7} M) still inhibited the pacemaker currents (n=5, Fig. 10A and B). Naproxen or L-NAME itself had no effect on pacemaker currents.

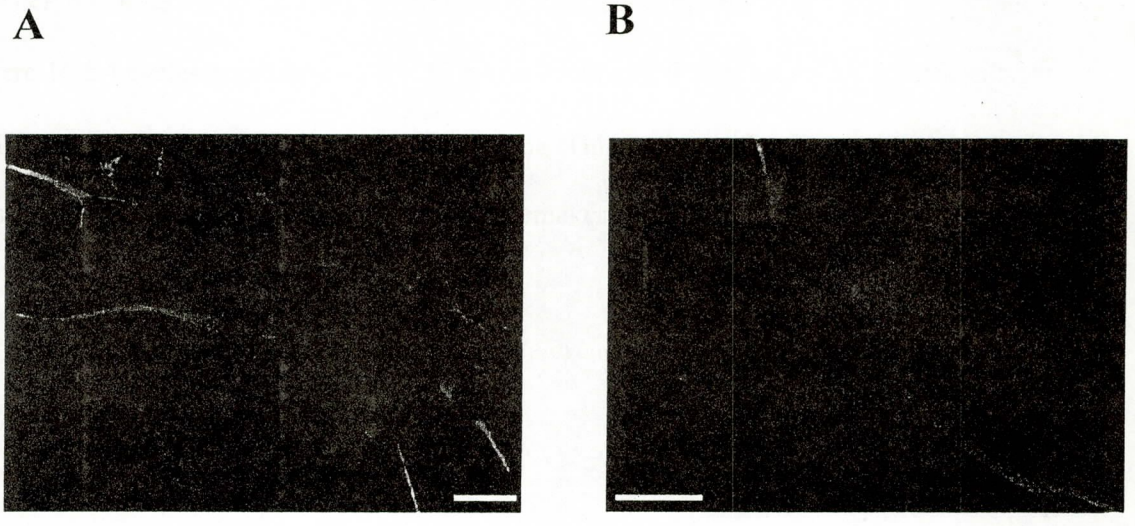

Fig. 1. Kit-immunoreactive ICC in cell culture. The tunica muscularis of the small intestine was digested with collagenase, and dispersed cells were cultured 2 days. Confocal laser microscopic image shows that the Kit-immunoreactive cells from networks (A), higher magnification of Kit-immunoreactive ICC in culture (B). Scale bars = 20 μ m in A and B.

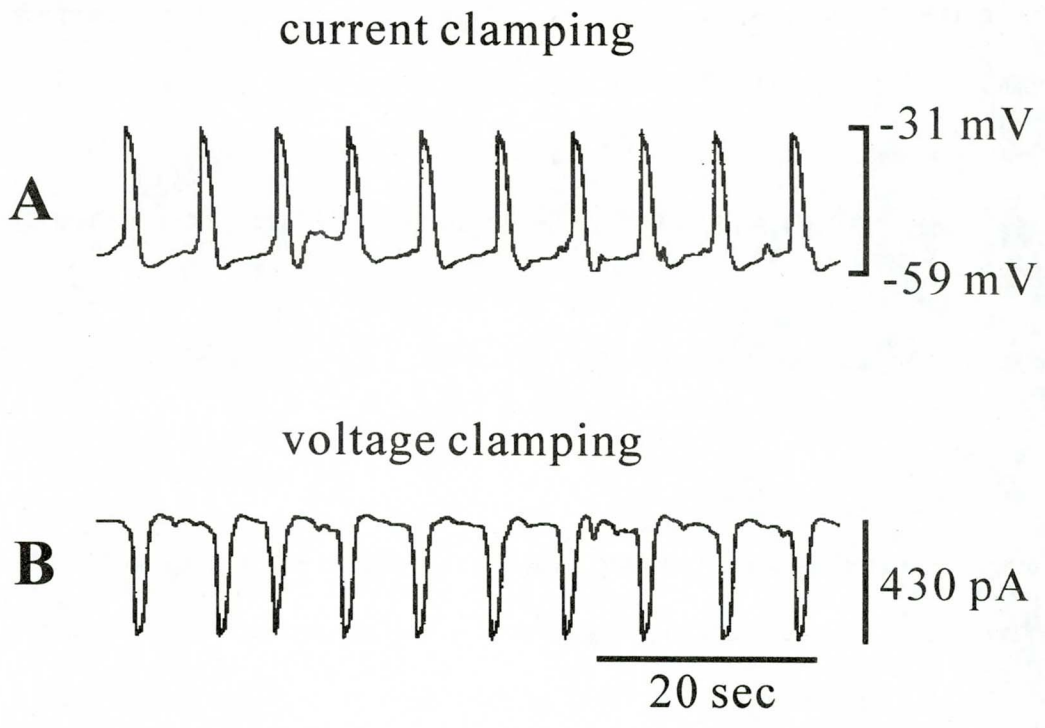

Fig. 2. Typical trace of pacemaker potentials in current clamping mode (A) and pacemaker currents in voltage clamping mode (B) recorded at a holding potential of -70 mV in cultured ICC from murine small intestine.

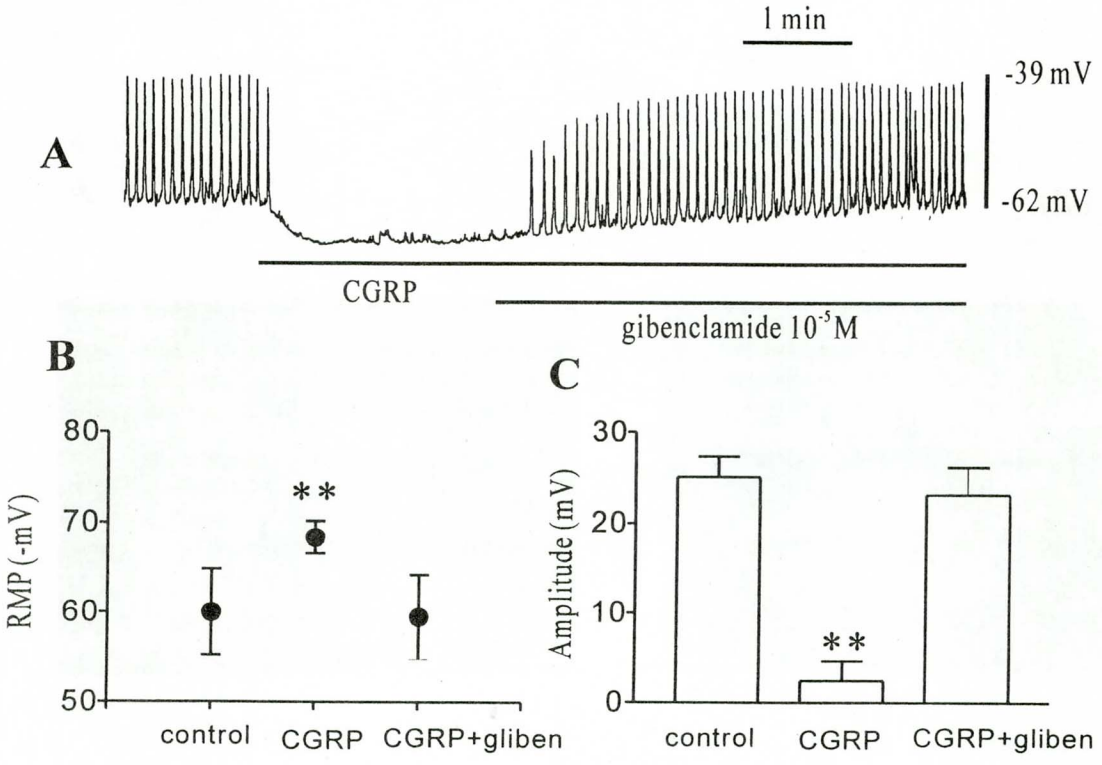

Fig. 3. Effects of CGRP on pacemaker potentials in cultured ICC from murine small intestine. Pacemaker potentials of ICC exposed to CGRP (5 x 10^{-7} M) in current clamping mode (I=0). The CGRP induced membrane hyperpolarization and decreased amplitude of pacemaker potential was reversed by glibenclamide (10^{-5} M) (A). Response to CGRP are summarized in B and C. Bars represent mean values ± SE. **($P<0.01$) Significantly different from the untreated control. GBC: glibenclamide.

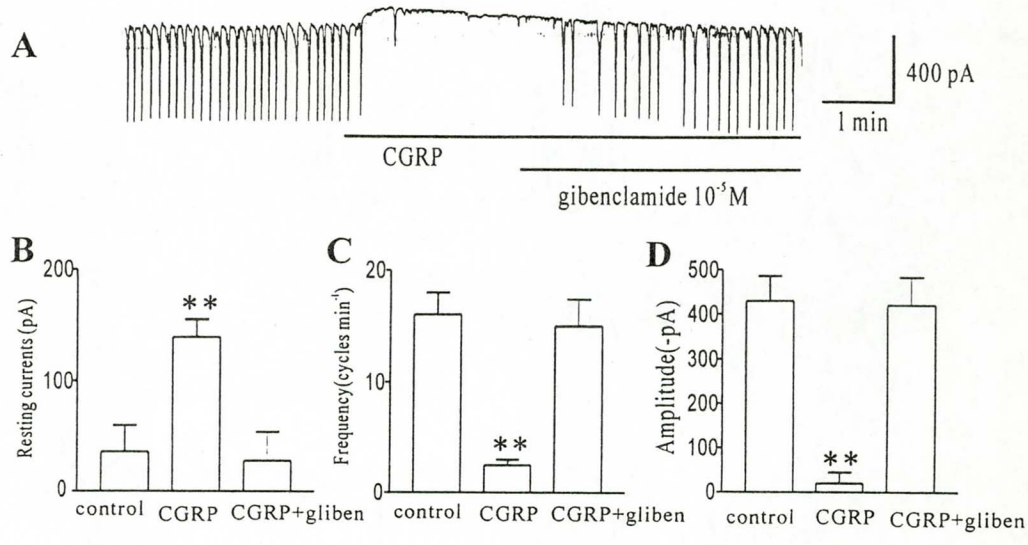

Fig. 4. Effects of CGRP on pacemaker currents in cultured ICC from murine small intestine. Pacemaker currents of ICC exposed to CGRP (5×10^{-7} M) in voltage clamping mode. The CGRP induced basal outward currents and decreased amplitude or frequency of pacemaker currents was reversed by glibenclamide (10^{-5} M) (A). Response to CGRP are summarized in B, C and D. Bars represent mean values ± SE. **($P<0.01$) Significantly different from the untreated control. GBC: glibenclamide.

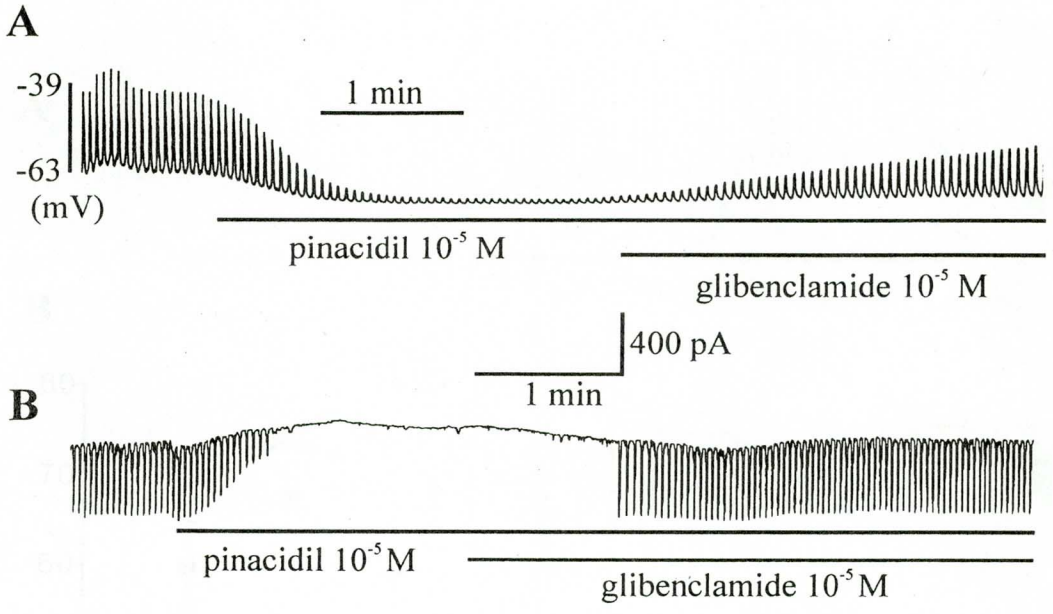

Fig. 5. Effects of pinacidil on pacemaker potentials and pacemaker currents in cultured ICC from murine small intestine. Pacemaker potentials of ICC exposed to pinacidil (10^{-5} M) in current clamping mode (I=0). The pinacidil induced membrane hyperpolarization and decreased amplitude of pacemaker potential was reversed by glibenclamide (10^{-5} M) (A). The pinacidil induced basal outward currents and decreased amplitude or frequency of pacemaker currents was reversed by glibenclamide (B).

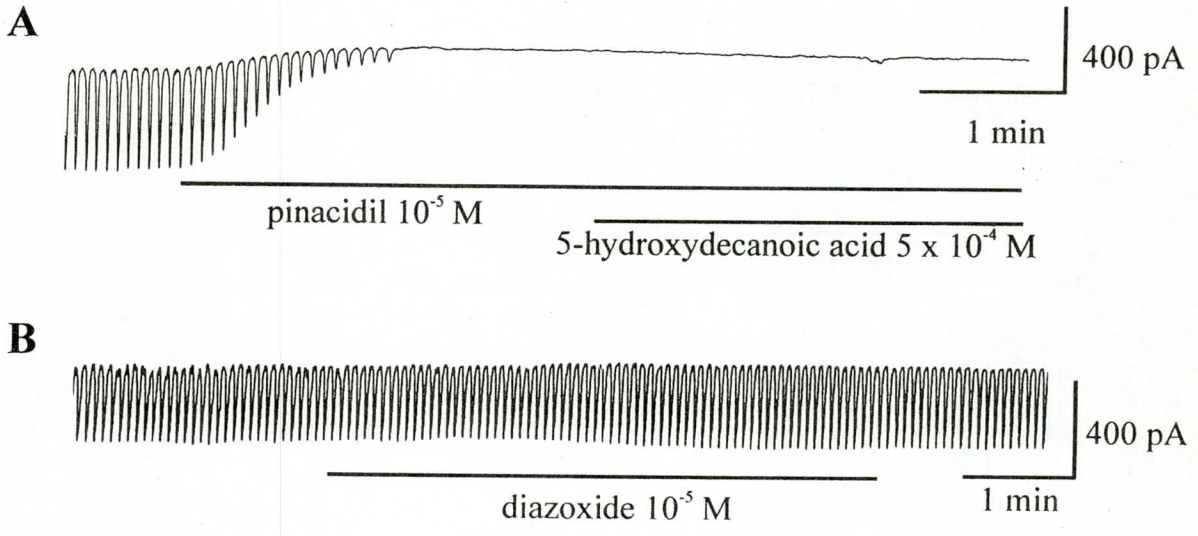

Fig. 6. Effects of 5-HD in pinacidil-induced responses and effects of diazoxide on pacemaker currents in cultured ICC from murine small intestine. The pinacidil induced basal outward currents and decreased amplitude or frequency of pacemaker currents was not reversed by 5-HD (A). Diazoxide had no effects on pacemaker currents (B). Bars represent mean values ± SE. **($P<0.01$) Significantly different from the untreated control.

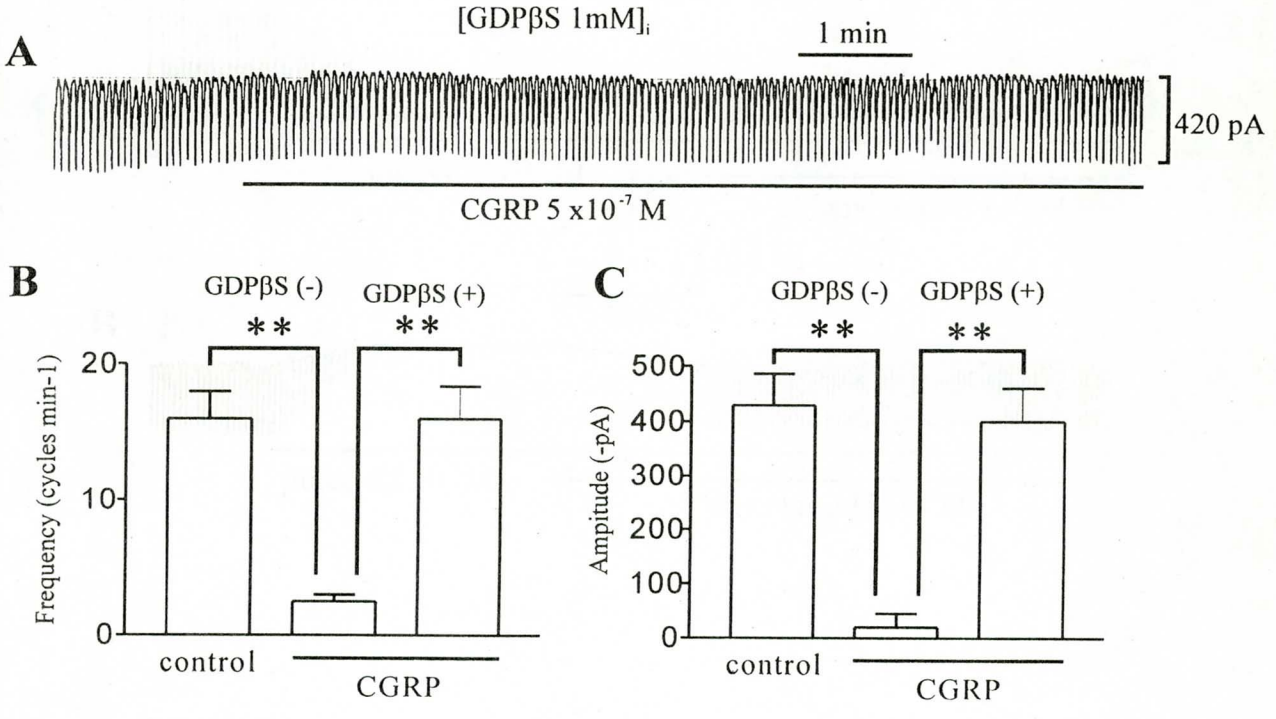

Fig. 7. Effects of GDP β S on the response to CGRP. Pacemaker currents of ICC exposed to CGRP (5 x 10^{-7} M) in the presence of GDP β S (1 mM) in the effects. GDP β S blocked the CGRP-mediated inhibition of pacemaker currents. The effect of CGRP in the presence of GDP β S but not in the pipette is summarized in B and C. Bars represent mean values ± SE. **($P<0.01$) Significantly different from the untreated control.

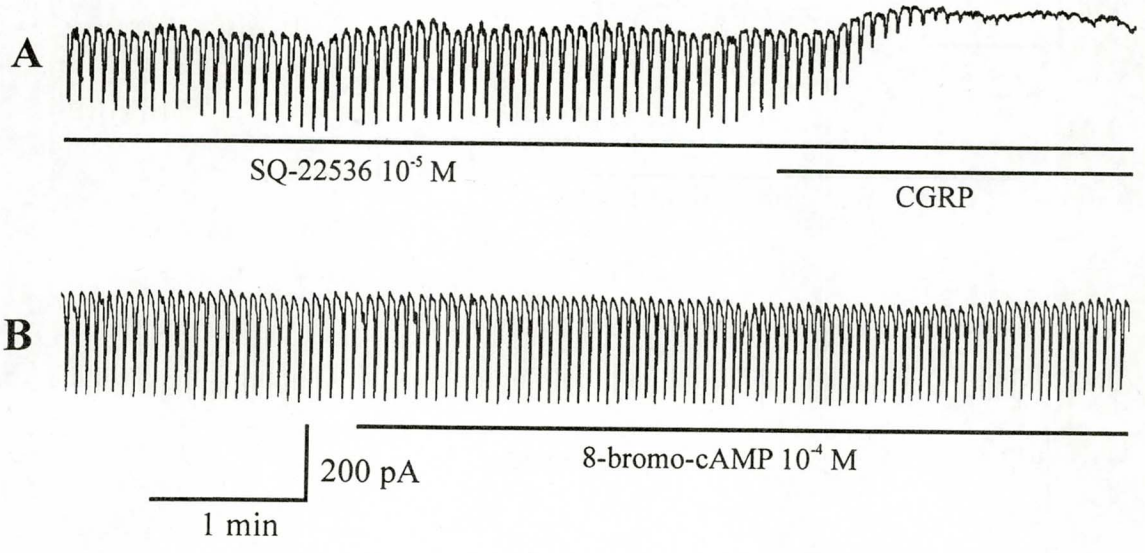

Fig. 8. Effects of adenylate cyclase inhibitor on the CGRP-mediated inhibition of pacemaker currents. (A) Effects of CGRP on pacemaker currents in the presence of SQ-22536 (10^{-5} M). SQ-22536 did not block the CGRP-mediated inhibition of pacemaker currents. (B) Effects of 8-bromo-cyclic AMP (10^{-4} M) on pacemaker currents. 8-bromo-cyclic AMP had no effect on pacemaker currents.

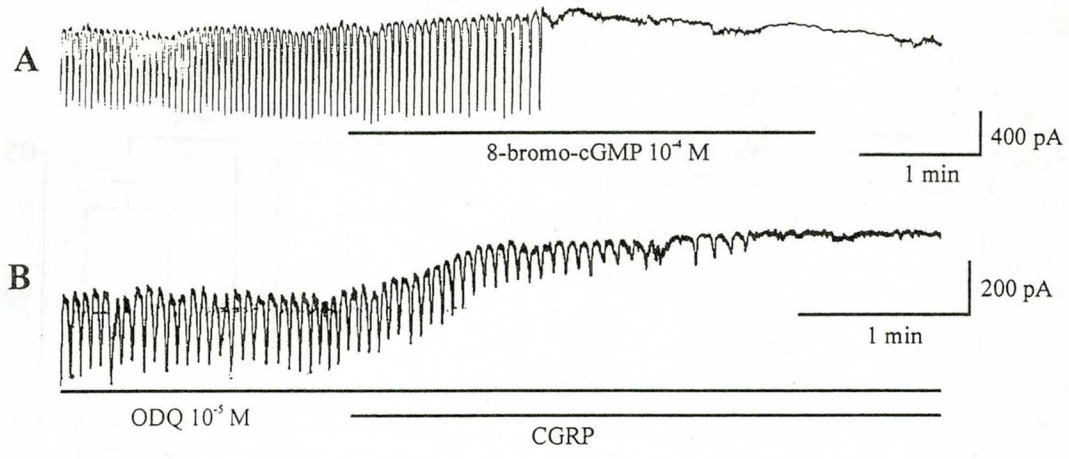

Fig. 9. Effects of guanylate cyclase inhibitor on the CGRP-mediated inhibition of pacemaker currents. (A) Effects of 8-bromo-cyclic GMP (10^{-4} M) on pacemaker currents. 8-bromo-cyclic GMP inhibited pacemaker currents. (B) Effect of CGRP in the presence of ODQ (10^{-5} M). ODQ did not block the CGRP-mediated inhibition of pacemaker currents.

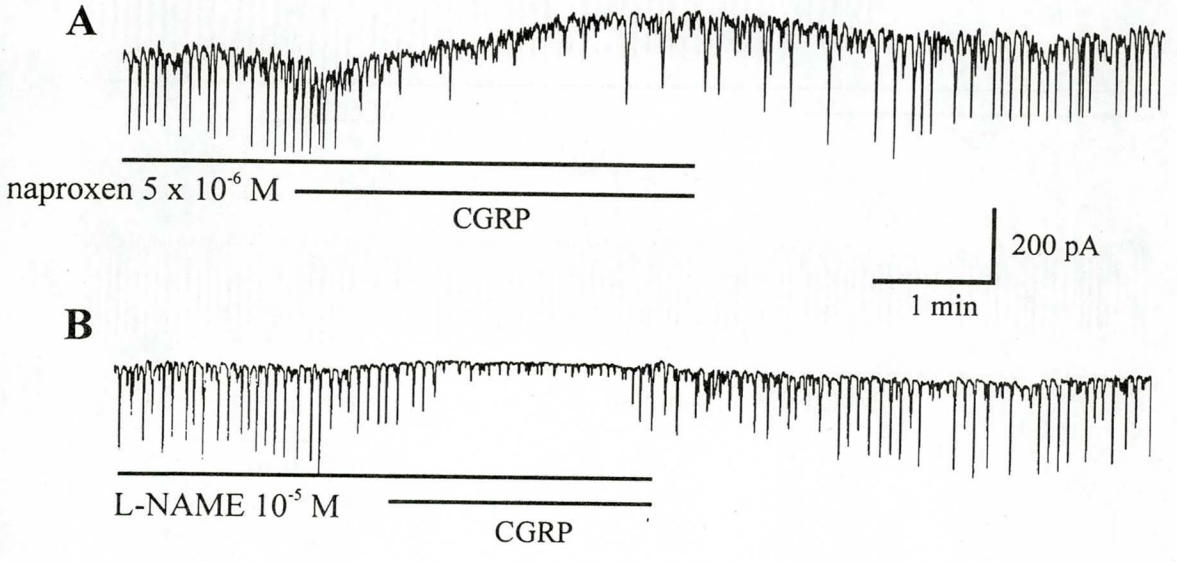

Fig. 10. Effects of cyclooxygenase or nitric oxide synthase inhibitor on the CGRP-mediated inhibition of pacemaker currents. (A) Naproxen (5 x 10^{-6} M) (a cyclooxygenase inhibitor) and (B) L-NAME (10^{-5} M) (a nitric oxide synthase inhibitor) did not block the CGRP-mediated inhibition of pacemaker currents.

4. Discussion

CGRP is a potent smooth muscle relaxant of vascular and gastrointestinal system. Various cellular events are involved in the smooth muscle, including activation of ATP-sensitive K^+ channels, activation of adenylate cyclase and adenosine 3',5'-cyclic monophosphate (cAMP) accumulation, generation of nitric oxide (NO) and guanosine 3',5'-cyclic monophosphate (cGMP) accumulation, and production of prostaglandins [24].

K^+ channels of smooth muscle regulate the membrane potential and cell excitability. ATP-sensitive K^+ channels stabilize the resting membrane potential. The activity of ATP-sensitive K^+ channels is regulated by cellular metabolism and provides a means of linking the electrical activity of a cell to its metabolic state. The activation of ATP-sensitive K^+ channels in vascular smooth muscle leads to membrane potential hyperpolarization and reduces the cellular electrical excitability, which cause vasodilation through closing voltage-dependent Ca^{2+} channels [25]. ATP-sensitive K^+ channels have also been reported in gastrointestinal smooth muscle cells and act as targets of neurotransmitters and peptides [26, 27]. Therefore the activity of ATP-sensitive K^+ channels is very important to regulate gastrointestinal motility. In intact gallbladder smooth muscle, CGRP induced prolonged hyperpolarization with cessation of spontaneous electrical activities that was sensitive to glibenclamide. Also, CGRP activated ATP-sensitive K^+ channels in single myocytes [28]. In this study CGRP also produced membrane hyperpolarization and inhibited the generation of pacemaker currents that was sensitive to glibenclamide in cultured ICC. Besides pinacidil showed

the mimicked action with CGRP on electrical activities in cultured ICC. Therefore, CGRP may acts on ATP-sensitive K^+ channels of ICC. There are two subtypes of ATP-sensitive K^+ channels in cardiac muscles, a sarcolemmal ATP-sensitive K^+ channels and mitochondrial ATP-sensitive K^+ channels. Mitochondrial ATP-sensitive K^+ channels are target by K^+ channel openers, which plays cardioprotective action in ischemia-reperfusion injury. Mitochondrial ATP-sensitive K^+ channels may be involved in mitochondrial Ca^{2+} handling [29]. In addition, pacemaker current in ICC is dependent on the periodic oscillation of intracellular Ca^{2+} between endoplasmic reticulum and mitochondria. The generation of pacemaker currents is abolished by xestospongin C, an inhibitor of IP_3-receptors in endoplasmic reticulum or by carbonyl cyanide m-chlorophenyl-hydrazone (CCCP), an inhibitor of proton pump in mitochondria. These findings suggest that pacemaker mechanism is initiated by release of Ca^{2+} from endoplasmic reticulum and followed to reuptake Ca^{2+} into the mitochondria [30]. Also, slow waves recorded in intact gastric smooth muscle are inhibited by 5-HD, an inhibitor of mitochondrial ATP-sensitive K^+ channels, suggesting mitochondrial ATP-sensitive K^+ channels involve in the generation of spontaneous electrical activity [31]. However, in this experiment, 5-HD did not block the CGRP-mediated responses and also, diazoxide (a mitochondrial ATP-sensitive K^+ channels opener) did not inhibit pacemaker currents, indicating that CGRP activated sarcolemmal ATP-sensitive K^+ channels in ICC.

Cyclic AMP- and cyclic GMP-dependent pathways provide the major inhibitory mechanisms that regulate GI muscles. A variety of inhibitory neurotransmitters and hormones elicit responses via enhanced production of cyclic AMP [16, 32]. In addition, the activation of ATP-sensitive K^+ currents by CGRP is mediated by elevation of cyclic AMP and subsequent stimulation of protein kinase A in the vascular and gallbladder

myocytes [33, 34]. Thus, cyclic AMP production seems to play a central role in the relaxant activity of CGRP on smooth muscle cells. However, SQ-22536 (an adenylate cyclase inhibitor) did not block the CGRP-mediated inhibition and cell permeable 8-bromo-cAMP had no effects the generation of pacemaker currents in cultured ICC. Already Jun et al. found that the inhibition of pacemaker current by β-adrenoceptor activation was not blocked SQ-22536 [19]. Generally it is well known that stimulating the β-adrenoceptors with catecholamines increase cyclic AMP levels by activating adenylate cyclase via G-protein [35]. Therefore, these results demonstrate that CGRP-mediated inhibition of pacemaker currents is not mediated by cyclic AMP production. These findings are consistent with previous observations of ATP-sensitive K^+ channels activation by cyclic AMP or protein kinase A –independent pathway in rat mesenteric vascular beds [36]. It has also been suggested that activation of ATP-sensitive K^+ channels can occur through GTP-binding proteins independent of the adenylate cyclase-proten kinase A pathway in cardiac myocytes. For example, exposure of preactivated Gi α-subunits to the cyclic surface of cardiac myocyte membrane patches resulted in increased KATP channel activity [37].

CGRP also relax smooth muscles in guinea-pig ileum and human colon by production of nitric oxide through the cyclic GMP production. Preincubation of L-NAME or L-N5(I-immunoethyl)ornithin, dihydrochloride(L-NIO), inhibitors of NOS, inhibited the relaxing effects of CGRP [38, 39]. In addition, pacemaker currents in cultured intestinal ICC are inhibited by 8-bromo-cyclic GMP [40] and SNAP, a NO donor, inhibited pacemaker currents and these effects were blocked by ODQ, a guanylate cyclase inhibitor [19]. However, in this study, preincubation of ODQ or L-NAME did not block

the CGRP-induced effects that suggests CGRP-mediated inhibition is not mediated by NO or cyclic GMP production. Also, it has been reported that CGRP relax guinea-pig and rat gastric smooth muscle by production of prostaglandins [4]. However, in this study, preincubation of naproxen, a cyclooxygenase inhibitor, did not block the CGRP-induced effects that suggests CGRP-mediated inhibition is not mediated by prostaglandins production.

In conclusion, the present findings CGRP inhibits the generation of pacemaker currents by activating of ATP-sensitive K^+ channels via G-protein dependent mechanism. Neither adenylate cyclase, guanylate cyclase, nitric oxide nor cyclooxygenase dependent pathways are involved in these effects. Therefore, it suggests that the modulation of electrical activities of ICC by CGRP may be a possible inhibitory mechanism in regulation of gastrointestinal motility.

5. Summary

ICC are pacemaker cells that activate the periodic spontaneous inward currents (pacemaker currents) responsible for the production of slow waves in gastrointestinal smooth muscle. The functional effects of calcitonin gene-related peptide (CGRP) on pacemaker currents in cultured ICC from murine small intestine were investigated using a whole-cell patch clamp technique at 30 °C. Under current clamping, ICC generated pacemaker potential and had a mean resting membrane potential of -58 ± 5 mV. Under voltage clamping, ICC produced pacemaker currents with a mean amplitude of -413 ± 61 pA and a mean frequency of 16 ± 2 cycles min^{-1}. Under voltage clamping at a holding potential of -70 mV, CGRP inhibited the amplitude and frequency of pacemaker currents and increased resting currents in the outward direction. These effects were blocked by intracellular GDP β S or glibenclamide, a specific ATP-sensitive K^+ channel blocker. In current clamping, CGRP hyperpolarized membrane and these effects were antagonized by glibenclamide, Pinacidil, an ATP-sensitive K^+ channel opener, also showed the mimicked effects with CGRP. Pinacidil produced membrane hyperpolarization in current clamping and inhibited the amplitude and frequency of pacemaker currents and increased resting currents in the outward direction in voltage clamping. These effects were blocked by glibenclamide. SQ-22536, an adenylate cyclase inhibitor did not block the CGRP-induced effects and cell permeable 8-bromo-cAMP had no effects on pacemaker currents. Neither L-NAME (an inhibitor of NOS), ODQ (an guanylate cyclase inhibitor) nor naproxen (a cyclooxygenase inhibitor) blocked the CGRP-induced effects. In conclusion CGRP inhibits the generation of

pacemaker currents by activating of ATP-sensitive K^+ channels via G-protein dependent mechanism. Neither adenylate cyclase, guanylate cyclase, nitric oxide nor cyclooxygenase dependent pathways are involved in these effects.

Key Words: ATP-sensitive K^+ channels; Calcitonin gene-related peptide; Interstitial cells of Cajal; Pacemaker currents; Small intestine

6. References

[1] Rasmussen TN, Schmidt P, Poulsen SS, Holst JJ. Localisation and neural control of the release of calcitonin gene-related peptide (CGRP) from isolated perfused porcine ileum. Regul Pept 2001;98:137-143.

[2] Holzer P. Calcitonin gene-related peptide. In J.H. Walsh and G.J.(Eds.), Dockray Gut Peptides, Biochemistry and Physiology, Raven Press, New York, 1994, pp. 493-523.

[3] Bartho L, Lemback F, Holzer P. CGRP is a potent relaxant of intestinal muscle. Eur J Pharmacol 1987; 135:449-451.

[4] Katsoulis S, Conlon JM. CGRPs relax guinea-pig and rat gastric smooth muscle. Eur J Pharmacol 1989; 161:129-134.

[5] Mulholland MW, Jaffer S. Stimulation of acetylcholine release in myenteric plexus by CGRP. Am J Physiol 1990; 259:G934-G939.

[6] Holzer P. Implications of tachykinin and calcitonin gene-related peptide in inflammatory bowel disease. Digestion 1998;59:269-283.

[7] Szurszwski JH. Electrical basis for gastrointestinal motility. In Physiology of the Gastrointestinal tract, 2nd, ed Johnson LR, pp. 383-422, 1987, Raven Press, New York.

[8] Ward SM, Burns AJ, Torihashi S, Sanders KM. Mutation of the proto-oncogene c-kit blocks development of interstitial cells and electrical rhythmicity in murine intestine. J Physiol 1994;480:91-97.

[9] Huizinga JD, Thunberg L, Kluppel M, Malysz J, Mikkelsen HB, Bernstein A. W/kit gene required for interstitial cells of Cajal for intestinal pacemake activity. Nature 1995;373:347-349.

[10] Sanders KM, Ordog T, Koh SD, Ward SM. Development and plasticity of

interstitial cells of Cajal. Neurogastroenterol Motil 1999;11:311-338.

[11] Sanders KM, Koh SD, Ordog T, Ward SM. Ionic conductances involved in generation and propagation of electrical slow waves in phasic gastrointestinal muscles. Neurogastroenterol Motil 2004;16:100-105.

[12] Sanders KM. A case for interstitial cells of Cajal as pacemakers and mediators of neurotransmission in the gastrointestinal tract. Gastroenterol 1996;111:492-515.

[13] Ward SM, Beckett EA, Wang X, Baker F, Khoyi M, Sanders KM. Interstitial cells of Cajal mediate cholinergic neurotransmission from enteric motor neurons. J Neurosci 2000;20:1393-1403.

[14] Vanderwinden JM, Rumessen JJ. Interstitial cells of Cajal in human gut and gastrointestinal disease. Microsc Res Tech 1999; 47:344-360

[15] Olsson C, Holmgren S. The control of gut motility. Comp Biochem Physiol 2001;128:481-503.

[16] Sanders KM. G protein-coupled receptors in gastrointestinal physiology IV. Neural regulation of gastrointestinal smooth muscle. Am J Physiol 1998;275:G1-G7.

[17] Kim TW, Koh SD, Ordog T, Ward SM, Sanders KM. Muscarinic regulation of pacemaker frequency in murine gastric interstitial cells of Cajal. J Physiol 2002; 546:415-425.

[18] Jun JY, Choi S, Yeum CH, Chang IY, Ho Jin You, Park CK, Kim MY, Kong ID, Kim MJ, Lee KP, So I, Kim KW. Substance P induces inward current and regulates pacemaker currents through tachykinin NK1 receptor in cultured interstitial cells of Cajal of murine small intestine. Eur J Pharmacol 2004; 495:35-42.

[19] Jun JY, Choi S, Yeum CH, Cang IY, Park CK, Kim MY, Kong ID, So I, Kim KW, You HJ. Noradrenalin inhibits pacemaker currents through stimulation of β1-

adrenoceptors in cultured interstitial cells of Cajal from murine small intestine. Br J Pharmacol 2004;141:670-677.

[20] Vannucchi MG. Receptors in interstitial cells of Cajal: identification and possible physiological roles. Microsc Res Tech 1999;47:325-335.

[21] Burnstock G, Lavin S. Interstitial cells of Cajal and purinergic signaling. Auto Neurosci 2002; 97:68-72.

[22] Thomsen L, Robinson TL, Lee JC, Farraway LA, Hughes MJ, Andrews DW, Huizinga JD. Interstitial cells of Cajal generate a rhythmic pacemaker current. Nature Med 1998;4:848-851.

[23] Koh SD, Sanders KM, Ward SM. Spontaneous electrical rhythmicity in cultured interstitial cells of Cajal from the murine small intestine. J Physiol 1998;513:203-213.

[24] Maggi, CA, Giuliani S, Zagorodnyuk V. Calcitonin gene-related peptide (CGRP) in the circular muscle of guinea-pig colon: role as inhibitory transmitter and mechanisms of relaxation. Regul Pept 1996;61:27-36.

[25] Quayl JM, Bonev AD, Brayden JE, Nelson MT. ATP-sensitive and inwardly rectifying potassium channels in smooth muscle. Physiol Rev 1997; 77:1165-1232.

[26] Jun JY, Kong ID, Koh SD, Wang XU, Perrino BA, Ward SM, Sanders KM. Regulation of ATP-sensitive K^+ channels by protein kinase C in murine colonic myocytes. Am J Physiol 2001; 281:C857-C864.

[27] Jun JY, Yeum CH, Yoon PJ, Cahng IY, Kim SJ, Kim KW. ATP-sensitive K^+ current and its modulation by substance P in gastric myocytes isolated from guinea-pig. Eur j Pharmacol 1998; 358:77-83.

[28] Zhang , Bonev AD, Nelson MT, Mawe GM. Activation of ATP-sensitive potassium currents in guinea-pig gall-bladder smooth muscle by neuropeptide CGRP. J Physiol

1994; 478:483-491.

[29] Mannhold R. KATP channel openers: Structure-activity relationships and therapeutic potential. Medicinal Research Rev 2004; 24:213-266.

[30] Ward SM, Ordog T, Koh SD, Baker SA, Jun JY, Amberg G, Monaghan K, Sanders KM. Pacemaking in interstitial cells of Cajal depends upon calcium handling by endoplasmic reticulum and mitochondria. J Physiol 2000;525:355-361.

[31] Kito Y, Suzuki H. Modulation of slow waves by hyperpolarization with potassium channel openers in antral smooth of the guinea-pig stomach. J Physiol 2003a;548:175-189.

[32] Ozaki H, Blondfield DP, Hori M, Sanders KM, Publicova NG. Cyclic AMP-mediated regulation of excitation-contraction coupling in canine gastric smooth muscle. J Physiol 1992;447:351-372.

[33] Quayle JM, Bonev AD, Brayden JE, Nelson MT. Calcitonin gene-related peptide activated ATP-sensitive K^+ currents in rabbit arterial smooth muscle via protein kinase A. J Physiol 1994;475:9-13.

[34] Zhang L, Bonev AD, Mawe GM, Nelson MT. Protein kinase A mediates activation of ATP-sensitive K^+ currents by CGRP in gallbladder smooth muscle. Am J Physiol 1994;267G494-G499.

[35] Bulbring E, Tomita T. Catecholamine action on smooth muscle. Pharmacol Rev 1987; 39:49-96.

[36] Randall MD, Mculloch AI. The involvement of ATP-sensitive potassium channels in β-adrenoceptor-mediated vasorelaxation in the rat isolated mesenteric arterial bed. Br J Pharmacol 1995; 115:607-612.

[37] Terzic A, Tung RT, Inanobe A, Katada T, Kurachi Y. G proteins activate ATP-

sensitive K^+ channels by antagonizing ATP-dependent gating. Neuron 1994; 12:885-893.

[38] Rekik M, Delvaux M, Frexinos J, Bueno L. The calcitonin gene-related peptide activates both cAMP and NO pathways to induce relaxation of circular smooth muscle cells of guinea-pig ileum. Peptides 1997;18:1517-1522.

[39] Boyer JC, Christen MO, Balmes JL, Bali JP. Calcitonin gene-related peptide-induced relaxation of isolated human colonic smooth muscle cells through different intracellular pathways. Biochem Pharmacol 1998; 56: 1097-1104.

[40] Koh SD, Kim TW, Jun JY, Glasgow NJ, Ward SM, Sanders KM. Regulation of pacemaker currents in interstitial cells of Cajal from murine small intestine by cyclic nucleotides. J Physiol 2000;527:149-162.