Characteristics of the Cholecystokinin-Induced Depolarization of Pacemaking Activity in Cultured Interstitial Cells of Cajal from Murine Small Intestine

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Abstract

Background/Aims: In this study, we studied the effects of cholecystokinin (CCK) on pacemaker potentials in cultured interstitial cells of Cajal (ICCs) from mouse small intestine using the whole cell patch clamp technique. Methods: ICCs are pacemaker cells that exhibit periodic spontaneous depolarization, which is responsible for the production of slow waves in gastrointestinal smooth muscle, and generate periodic pacemaker potentials in current-clamp mode. Results: Exposure to CCK (100 nM-5 µM) decreased the amplitudes of pacemaker potentials and depolarized resting membrane potentials. To identify the type of CCK receptors involved in ICCs, we examined the effects of CCK agonists and found that the addition of CCK1 agonist (A-71323, 1 µM) depolarized resting membrane potentials, whereas exposure to CCK2 agonist (gastrin, 1 µM) had no effect on pacemaker potentials. To confirm these results, we examined the effects of CCK antagonists and found that pretreatment with CCK1 antagonist (SR 27897, 1 µM) blocked CCK-induced effects. However, pretreatment with CCK2 antagonist (LY 225910, 1 µM) did not. Furthermore, intracellular GDPβS suppressed CCK-induced effects. To investigate the involvements of phospholipase C (PLC), protein kinase C (PKC), and protein kinase A (PKA) in the effects of CCK in cultured ICCs, we used U-73122 (an active PLC inhibitor), chelerythrine (a PKC inhibitor), SQ-22536 (an inhibitor of adenylate cyclase), or mPKAI (an inhibitor of myristoylated PKA). All inhibitors blocked the CCK-mediated effects on pacemaker potentials. In addition, we found that transient receptor potential classical 5 (TRPC5) channel was involved in CCK-activated currents in cultured ICCs. Conclusion: These results suggest that the CCK induced depolarization of pacemaking activity occurs in a G-protein-, PLC-, PKC-,
Introduction

Cholecystokinin (CCK) was one of the first gastrointestinal (GI) hormones discovered, and is produced in specialized epithelial cells located in the mucosa of the small intestine [1, 2]. The structural characterization of CCK and gastrin [3, 4], the pharmacological identification [5-9] and cloning [10, 11] of CCK and gastrin receptors, the characterization of receptor location, the characterizations of peptide and receptor genes, and developments of receptor antagonists and receptor/agonist knockout animals [12-15] have led to important advancements in our understanding of the physiological and pathophysiological roles of CCK and of gastrin signaling [16]. Two CCK receptors, CCK₁ and CCK₂, have been identified, and it has been well established that CCK₁ and CCK₂ regulate a number of physiological functions, such as, gallbladder contraction, pancreatic enzyme release, gastric acid secretion, and pyloric sphincter closure [17,18]. Both CCK₁ and CCK₂ mediate the contraction of guinea pig ileum, whereas guinea pig gallbladder contraction is mediated solely by CCK₁.

Interstitial cells of Cajal (ICCs) are the pacemaker cells of the GI system and have multifunctional roles. ICCs generate rhythmic oscillations in membrane potential, known as slow waves [19-21]. Furthermore, the discovery that ICCs express c-Kit, the proto-oncogene [22] that encodes the receptor tyrosine kinase Kit, offers an immunohistochemical means of determining the structure and distribution of ICC networks. The absence of or low numbers of ICCs causes abnormally slow electrical waves and reduces smooth muscle cell contractility and intestinal transit. In addition, the loss of ICCs is implicated in variable motility disorders, which indicates that ICCs play an important role in the regulation of GI motility [23]. In addition, evidence indicates that endogenous agents, such as, neurotransmitters, hormones, and paracrine substances modulate GI tract motility by influencing ICCs.

Therefore, in this study, we investigated the possibility that CCK affects the electrical properties of cultured ICCs, and characterized the CCK receptor subtypes involved.

Materials and Methods

Preparation of cells and cell cultures

Animal care and experiments on animals were conducted in accordance with the principles issued by the ethics committee of Pusan National University (Republic of Korea). Balb/c mice were used in the studies. 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 using Krebs-Ringer bicarbonate solution, and the tissues obtained were pinned to the base of a Sylgard dish. Mucosa was then removed by sharp dissection. Small tissue strips of intestine muscle (consisting of both circular and longitudinal muscles) were equilibrated in Ca²⁺-free Hank’s solution (containing, in mM: KCl 5.36, NaCl 125, NaOH 0.34, Na₂HCO₃ 0.44, glucose 10, sucrose 2.9 and HEPES 11, pH 7.4) for 30 min. Cells were then dispersed in an enzyme solution containing collagenase (Worthington Biochemical, Lakewood, NJ, U.S.A., 1.3 mg ml⁻¹), bovine serum albumin (BSA, Sigma-Aldrich, St Louis, MO, U.S.A., 2 mg ml⁻¹), trypsin inhibitor (Sigma-Aldrich, 2 mg ml⁻¹), and ATP (0.27 mg ml⁻¹). Cells were then plated onto sterile glass coverslips coated with murine collagen (2.5 µg ml⁻¹; Falcon/BD, Franklin Lakes, NJ, U.S.A.) in a 35 mm culture dish, and cultured at 37°C in a 95% O₂-5% CO₂ incubator in smooth muscle growth medium (SMGM; Clonetics, San Diego, CA, U.S.A.) supplemented with 2% antibiotics/antimycotics (Gibco, Grand Island, NY, U.S.A.) and murine stem cell factor (SCF; 5 ng ml⁻¹; Sigma-Aldrich). All experiments on single cells were performed on cells cultured for 1 day. ICCs were identified immunologically using anti-c-kit antibody (phycoerythrin (PE)-conjugated rat anti-mouse c-kit monoclonal antibody; eBioscience, San Diego, CA) at a dilution of 1:50 for 20 min.
Patch-clamp experiments

The physiological salt solution used to bathe cultured ICC cells (Na⁺-Tyrode) contained (in mM): KCl 5, NaCl 135, CaCl₂ 2, glucose 10, MgCl₂ 1.2, and HEPES 10, adjusted to pH 7.4 with NaOH. Cs⁺-rich external solution was made by replacing NaCl and KCl with equimolar CsCl. The pipette solution used to examine pacemaking activity contained (in mM): KCl 140, MgCl₂ 5, K₂ATP 2.7, NaGTP 0.1, creatine phosphate disodium 2.5, HEPES 5, and EGTA 0.1 (adjusted to pH 7.2 with KOH). The pipette solution for TRPC5 channels contained (in mM): CsCl 140, HEPES 10, Tris-GTP 0.5, EGTA 0.5, and Mg-ATP 3 (adjusted to pH 7.3 with CsOH). Patch-clamp techniques were conducted in whole-cell configuration to record membrane currents (voltage clamp) and potentials (current clamp) from cultured ICCs using Axopatch 1-D and Axopatch 200B amplifiers (Axon Instruments, Foster, CA). Command pulses were applied using an IBM-compatible personal computer and pClamp software (version 6.1 and version 10.0; Axon Instruments). Data were filtered at 5kHz and displayed on an oscilloscope, a computer monitor, and/or a pen recorder (Gould 2200; Gould, Valley View, OH, USA). Results were analyzed using pClamp and Origin software (version 6.0, Microcal, USA). All experiments were performed at 30–33°C.

Immunohistochemistry

Cultured ICCs from the small intestines of Balb/C mice were used for immunohistochemistry. Cultured ICCs were fixed in cold acetone (4 °C) for 5 min, washed in phosphate-buffered saline (PBS; 0.01 M, pH 7.4), and immersed in 0.3% Triton X-100 in PBS. After blocking with 1% BSA in 0.01 M PBS for 1 hour at room temperature, cells were incubated with a rat monoclonal antibody raised against c-Kit (Ack2; ebioscience) at 0.5 μg/ml or with a rabbit polyclonal antibody against CCK₁ or CCK₂ in PBS for 24 hours (4°C). After rinsing in PBS at 4°C, cells were labeled with fluorescein isothiocyanate (FITC)-coupled donkey anti-rabbit IgG secondary antibody (1:100; Jackson Immunoresearch Laboratories, Bar Harbor, MN, U.S.A.) or Texas red-conjugated donkey anti-rat IgG (1:100, Jackson Immunoresearch Laboratories) for 1 hour at room temperature. For double immunostaining, specimens were incubated with a mixture of antibodies raised against CCK₁ or CCK₂ and antibody raised against c-kit for 24 h at 4°C. After thorough washing with PBS, the mixture of labeled secondary antibodies was incubated for 1 hour at room temperature. Cells were examined under an FV 300 laser scanning confocal microscope (Olympus, Tokyo) at an excitation wavelength appropriate for FITC (495 nm) or Texas red (590 nm). Final images were constructed using Flow-View software (Olympus).

Statistical analysis

Data are expressed as means±standard errors. The significances of differences between results were evaluated using the Student’s t-test. P-values of < 0.05 were deemed significant. The n values reported in the text refer to the number of cells used in patch-clamp experiments.

Results

Effects of CCK on pacemaking activity in cultured ICC clusters

In current clamp mode, cells in cultured ICC clusters had a mean resting membrane potential of -59 ± 3 mV and produced electrical pacemaking activity of frequency 15 ± 3 cycles per minute and amplitude 26 ± 4 mV (n = 65) at 30°C. We first examined the effect of CCK on pacemaking activity. CCK (100 nM–5 μM) decreased amplitude and induced the depolarization of pacemaking activity in a concentration-dependent manner (Fig. 1); mean amplitudes were by 25.5 ± 1.2 mV at 100 nM (n = 4), 26.1 ± 0.5 mV at 500 nM (n = 5), 10.7 ± 0.6 mV at 1 μM (n = 5), and 3.82 ± 0.4 mV at 5 μM (n = 4; Fig. 1E), and corresponding depolarization were 3.75 ± 0.4 mV at 100 nM (n = 4), 6.12 ± 0.5 mV at 500 nM (n = 5), 16.31 ± 0.4 mV at 1 μM (n = 5), and 26.25 ± 0.6 mV at 5 μM (n = 4; Fig. 1F). These results suggested that CCK decreased amplitude and induced the depolarization of pacemaking activity in a dose-dependent manner in ICCs.
CCK₁ receptor was involved in the CCK-induced depolarization of pacemaking activity

To determine whether the regulatory effects of CCK are mediated by CCK₁ or CCK₂ receptors, we examined the effects of CCK₁ and CCK₂ agonists. It was found that a CCK₁ agonist (A-71623 1 μM) depolarized pacemaking activity (Fig. 2A) but that a CCK₂ agonist (Gastrin I 1 μM) had no effect (Fig. 2B). Mean depolarization was 25.28 ± 0.8 mV for A-71623
(n = 6), and 1.75 ± 0.7 mV for Gastrin I (n = 6; Fig. 2C). Furthermore, pretreatment with a CCK₁ antagonist (SR27897 1 μM) (n = 7, Fig. 3A) for 10 min blocked CCK-induced depolarization of pacemaking activity. However, pretreatment with a CCK₂ antagonist (LY225910 1 μM) (n = 7, Fig. 3B) did not. Mean depolarization was 1.25 ± 0.6 mV for SR27897 (n = 7), and 26.12 ± 0.7 mV for LY225910 (n = 7; Fig. 3C). In addition, we checked for the presence of CCK₁ and CCK₂ receptors by immunolabeling in cultured ICCs. The co-localization of CCK₁ and c-kit immunoreactivities were co-localized in cultured ICCs. The mixed color (yellow) indicates the co-localization of CCK₁ and c-kit immunoreactivities. Scale bars: 10 µm.

**Fig. 3.** Effects of CCK receptor antagonists on pacemaking activity in cultured ICC clusters. (A) Effects of SR27897 (CCK₁ antagonist) on CCK-induced effects on pacemaking activity. SR27897 blocked the CCK-induced depolarization of pacemaking activity. (B) Effects of LY225910 (CCK₂ antagonist) on CCK-induced effects on pacemaking activity. LY225910 did not inhibit the CCK-induced depolarization of pacemaking activity. (C) Responses to CCK antagonists are summarized. Bars represent mean values ± SE. **P<0.01: significantly different from the control.

**Fig. 4.** Expressions of CCK₁ and CCK₂ proteins in cultured ICCs. (A) Double-labeling of cultured ICCs with CCK₁ (green) and c-kit (red) antibodies. Cultured ICCs showed the co-localization of CCK₁ and c-kit immunoreactivities. The mixed color yellow (arrows) indicates the co-localization of CCK₁ and c-kit immunoreactivities. (B) Double-labeling of cultured ICCs with CCK₂ (green) and c-kit (red) antibodies. CCK₂ and c-kit immunoreactivities were co-localized in cultured ICCs. The mixed color (yellow) indicates the co-localization of CCK₂ and c-kit immunoreactivities.
ICCs from murine small intestine showed that these proteins were localized in ICCs. These results suggest that CCK functions in ICCs via CCK<sub>1</sub> receptors.

Effects of external Ca<sup>2+</sup>-free solution and of Ca<sup>2+</sup>-ATPase inhibitor in endoplasmic reticulum on the CCK-induced depolarization of pacemaking activity

External Ca<sup>2+</sup> influx is necessary for GI smooth muscle contractions and is essential for the generation of pacemaking activity by ICCs. Furthermore, the generation of pacemaking activity is known to be dependent on intracellular Ca<sup>2+</sup> oscillations [24]. To study the roles of external and internal Ca<sup>2+</sup>, CCK was investigated under external Ca<sup>2+</sup>-free conditions and in
the presence of thapsigargin, an inhibitor of Ca\(^{2+}\)-ATPase in the endoplasmic reticulum [25, 26]. In the presence of an external Ca\(^{2+}\)-free solution, pacemaking activity was completely abolished, and CCK-induced depolarizations were lower than in Ca\(^{2+}\)-containing solutions (n = 7; Fig. 5A). In addition, in the presence of thapsigargin, pacemaking activity was also completely abolished and CCK-induced effects were significantly inhibited (n = 7; Fig. 5B). Mean depolarization was 10.3 ± 2.1 mV for external Ca\(^{2+}\)-free solutions, and 1.2 ± 0.7 mV with thapsigargin (Fig. 5C). These results suggest that Ca\(^{2+}\) release from intracellular stores is a major mechanism responsible for the CCK-induced depolarization of pacemaking activity.

**Effects of G proteins on CCK-induced depolarization of pacemaking activity**

To investigate the roles played by G proteins during the CCK-induced depolarization of pacemaking activity, we applied GDP\(_{\beta}\)S (a non-hydrolysable guanosine 5′-diphosphate analogue that permanently inactivates G-protein binding proteins [27]) using patch pipettes. When GDP\(_{\beta}\)S (1 mM) was in the pipette solution, CCK-induced depolarizations were lower than under GDP\(_{\beta}\)S-free conditions (n = 6; Fig. 6A). Mean depolarization was 11.75 ± 1.3 mV in the presence of GDP\(_{\beta}\)S (Fig. 6B). These results suggest that G-protein stimulation is required for CCK-induced depolarization.

**Effects of phospholipase C-, protein kinase C-, and protein kinase A-inhibitors on the CCK-induced depolarization of pacemaking activity**

Because CCK-induced depolarization is related to intracellular Ca\(^{2+}\) mobilization, we investigated whether CCK-induced effects required phospholipase C (PLC) activation. Accordingly, CCK-induced depolarizations were measured in the presence of U-73122 (an active PLC inhibitor [28]). Pacemaking activity was completely abolished by U-73122 (5 μM), and under these conditions, CCK-induced depolarizations were suppressed (n = 6; Fig. 6A). Mean depolarization was 11.75 ± 1.3 mV in the presence of GDP\(_{\beta}\)S (Fig. 6B). These results suggest that G-protein stimulation is required for CCK-induced depolarization.

**Effects of U-73122 (a phospholipase C inhibitor) and of chelerythrine (a protein kinase C inhibitor) on CCK-induced pacemaking activity depolarization in cultured ICC clusters. (A) Pacemaking activities of ICCs exposed to CCK in the presence of U-73122 (5 μM). U-73122 blocked the CCK-induced pacemaking activity depolarization. (B) Pacemaking activity of ICCs exposed to CCK in the presence of chelerythrine (1 μM). Chelerythrine blocked the CCK-induced depolarization of pacemaking activity. (C) Responses to CCK in the presence of U-73122 or chelerythrine are summarized. Bars represent mean values ± SEs. **P<0.01: significantly different from the control.
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The effects of cholecystokinin (CCK) on intercalated disk cells (ICC) were investigated. CCK-induced depolarization of pacemaking activity in cultured ICC clusters was blocked by the PLC inhibitor SQ-22536 (10 μM) and the PKA inhibitor mPKAI (1 μM). CCK activation of inward currents and its current-voltage (I-V) relationship were determined using the whole cell patch-clamp technique. CCK (10 μM) induced inward currents, and the I-V relationships showed a typical doubly rectifying shape. These results suggest that the CCK-induced depolarization of pacemaking activity occurs in a PLC-, PKC-, and PKA-dependent manner.
Involvement of TRPC5 channels in CCK-activated currents (I_{CCK}) in cultured ICCs

It has been reported that CCK activates nonselective cation channels (NSCCs) [30-32], and thus we sought to identify the NSCCs involved in CCK-activated current. CsCl-rich solutions were used in the pipette and bath to record I_{CCK}. Under voltage clamp conditions at a holding potential of -60 mV, CCK (10 μM) induced I_{CCK} (n = 13, Fig. 9A). To determine the current-voltage (I-V) relationship, we applied a ramp pulse from +100 mV to −100 mV for 1 s. Whole cell currents were recorded under the condition 140 mM [Cs]o and [Cs]i, as a control for subtraction purposes to obtain the I-V relationship of I_{CCK}. The I-V relationship (Fig. 8B; b-o) obtained by subtracting current in the absence of CCK (Fig. 9A; a) from that in the presence of CCK (Fig. 9A; b) was similar to that of overexpressed TRPC5 (transient receptor potential classical 5) in HEK 293 cells [33, 34]. These results suggest that TRPC5 channel is a candidate for CCK induced inward currents in cultured ICCs from murine small intestine.

Discussion

In this study, we found that CCK inhibited pacemaker activity amplitudes of ICCs and depolarized resting membrane potentials via CCK_i. We also found PLC, PKC, and PKA mediate the inhibition of ICC pacemaker potential by CCK, and that TRPC5 channel is a potential candidate for current activation by CCK in cultured ICCs from murine small intestine.

In humans, strong evidence suggests that CCK_i activation is involved in the regulation of numerous physiological processes, including gallbladder contraction, relaxation of the sphincter of Oddi, stimulation of pancreatic secretion, slowing of colonic motility, regulation of satiety, reduced esophageal sphincter relaxation, and in the inhibitions of gastric emptying and acid secretion [35-46]. Although several authors have shown that the CCK_i is relevant in various GI diseases, the role played by CCK_i under these conditions has not been firmly established [35, 47]. Others have suggested that CCK_i could be involved in various GI motility pathologies, such as, gall bladder disease, irritable bowel syndrome, functional dyspepsia, chronic constipation, and gastroesophageal reflux disease [39, 48, 49]. Several GI tissues express CCK_i, CCK_o or both, and importantly, the tissue distributions of CCK_i and CCK_o exhibit relevant inter-species variations, which means that results from cultured cells from murine small intestine studies cannot always be extrapolated to humans [50-52]. CCK and gastrin were among the first GI hormones discovered. However, their physiological roles and clinically relevant roles in GI diseases remain unclear and even controversial [53-55].

Numerous studies have shown that several signaling molecules modulate calcium oscillations induced by CCK_i, Goα, Gα11, and Gα14 and the β and γ subunits probably released from Gq family members, play important mediator roles in oscillatory calcium response [56, 57]. The frequencies of calcium oscillations is also regulated by the phosphorylations of IP_3 receptors in response to physiological doses of CCK via a mechanism dependent on the PKA pathway [58, 59]. In addition to the β- and γ-PLC isoforms, two other phospholipases are activated by CCK receptors [60, 61], and also several papers have described the involvements of PKCs in both CCK_i and CCK_o signaling using broad spectrum PKC inhibitors. More recently, the activation of several PKC isoforms by gastrin and CCK has been reported [62, 63]. Although both CCK_i and CCK_o activate the PLC pathway via a Gα_{11/14} protein, only CCK_i is coupled to Gq. In pancreatic acinar cells, CCK induces adenylate cyclase activity and in CHO cells stably transfected with CCK_o, high doses of CCK increased intracellular cAMP by stimulating this enzyme [57]. In the gallbladder, ICCs are in intimate contact with smooth muscle cells via gap junctions, and are responsible for the generation of smooth muscle rhythmic activities. The finding that gallbladder ICCs strongly express CCK_i suggests CCK-induced gallbladder activity. Recently, Gong et al.[64] suggested CCK increases [Ca^{2+}]_i in ICCs via CCK_i receptor and that this effect depends on the release of IP_3 R-operated Ca^{2+} stores, which are negatively regulated by the PKC-mediated phosphorylation of IP_3_R.

In this study, CCK inhibited pacemaker potentials in a PLC, PKC, and PKA dependent manner through CCK_i, and CCK_o was expressed in cultured ICCs. Therefore, we believe that
CCK$_1$ has an important role in GI motility. However, the role of CCK$_2$ in GI motility requires further investigation.

Recently, it has been suggested that CCK activates TRPC channels in amygdaloid [30] and entorhinal [31] neurons, and Grisanti et al. [32] suggested the activation of CCK2 receptors robustly potentiates the function of TRPC5 channels in HEK293 cells. Therefore, we investigated the molecular candidates for CCK channel activation in ICCs, and found that TRPC5 was involved in CCK channel activation in murine small intestine ICCs. However, Si et al. [65] suggested that hyperpolarization-activated cyclic nucleotide-gated (HCN) channels are present in ICCs in the murine gastric antrum and that they might be an important regulator of ICC excitability and pacemaker activity. Also, extracellular calcium might trigger the activation of HCN channels by CCK in cultured ICCs. In the GI tract, TRPC5 and HCN channels could play important roles in GI motility. Additional investigatory studies are required to determine the relations and characteristics of both channels in ICCs of the GI tract.

The motor action of GI smooth muscle is initiated by periodic membrane depolarization, which gives rise to slow waves. Slow waves play an important role in the regulation of GI motility by determining the frequency and timing of smooth muscle contractions. ICCs are pacemaker cells that generate slow waves by producing spontaneous pacemaker potentials, and are connected to each other to form a network and form gap junctions with smooth muscle cells. Accordingly, pacemaker potentials generated by ICCs are directly transmitted to smooth muscle through gap junctions [19-21]. In addition, ICCs mediate inhibitory and excitatory signals from the enteric nervous system to smooth muscle, and thus, play an important role in the determination and regulation of GI motility. Furthermore, it has been reported that ICCs express muscarinic, adrenergic, tachykinin, somatostatin and purinergic receptors, which suggests that they are the targets of a variety of endogenous substances.

In this study, we focused on the roles of CCK and of its receptors (CCK$_1$) in ICCs from murine small intestine. This study demonstrates that investigations into the roles of CCK and CCK signaling in cultured ICCs from murine small intestine have led to important advancements in our understanding of the physiological and pathophysiological roles of CCK signaling. Furthermore, the involvement of CCK$_1$ in cultured ICCs suggests that these cells mediate the effects of circulating hormones on smooth muscle activity in addition to generating slow wave pacemaker activity and mediating the effects of enteric neurotransmitters.

In conclusion, CCK was found to induce the depolarization of pacemaking activity in a G-protein-, PLC-, PKC-, and PKA-dependent manner via CCK$_1$ receptor. Also, the study suggests that TRPC5 is a candidate for CCK-activated inward currents in cultured ICCs from murine small intestine. Therefore, the ICCs are targets for CCK and their interaction can affect intestinal motility.

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