

RAPID COMMUNICATION

## Control of gallbladder contractions by cholecystokinin through cholecystokinin-A receptors on gallbladder interstitial cells of cajal

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### Abstract

**AIM:** To identify the cholecystokinin (CCK)-A receptors (CCK-AR) on the guinea pig gallbladder interstitial cells of cajal (ICC) and to study CCK-8 induced gallbladder muscle strip contractions through the CCK-AR.

**METHODS:** The existence of CCK-AR was examined by immunohistofluorescence on sectioned tissue and cultured cells. *In vitro* contractile response of guinea pig gallbladder muscle strips and the strips with ICC removed were also studied with CCK-8 receptors added.

**RESULTS:** In tissue sections, intensely CCKAR-immunoreactive interstitial cells were found mainly in the muscular layers. In cultured cell sections, distinctive double staining of C-kit and CCK-AR ICCs were found. When we removed the ICC of the gallbladder, CCK-8 induced muscle strip contraction dose response curve significantly shifted to the right.

**CONCLUSION:** We proved that both the existence of CCK-AR on the guinea pig gallbladder ICC and CCK evoked contraction are mediated through direct action on CCK-AR on the gallbladder ICC.

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**Key words:** Gallbladder; Interstitial cells of cajal; Cholecystokinin-A receptor; C-kit; Immunohistofluorescence

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### INTRODUCTION

Cholecystokinin (CCK) is released from mucosal endocrine cells in the proximal small intestine in response to a meal<sup>[1]</sup>, and it is classically known to stimulate the gallbladder contraction. The CCK-induced gallbladder contractions were once believed to be brought about by direct stimulation of gallbladder smooth muscle. The CCK receptors have been identified by using isolated gallbladder muscle membranes and autoradiographical analysis<sup>[2-4]</sup>. Two CCK receptors, CCK-A (CCK-AR) and CCK-B receptors (CCK-BR) have been identified<sup>[5]</sup>. It is well established that CCK-AR and CCK-BR regulate a number of physiological functions, e.g. gallbladder contraction<sup>[6]</sup>, pancreatic enzyme release<sup>[7]</sup>, gastric acid secretion<sup>[8]</sup> and pyloric sphincter closure<sup>[9,10]</sup>. In 1892, a special kind of cell which is now called "interstitial cells of Cajal" (ICC) in the gastrointestinal tract was first described by the famous Spanish neuro-histologist Ramon y Cajal<sup>[11]</sup>. It has been approved that ICC play an important role in gastrointestinal tissues by generating and propagating electrical slow waves to gastrointestinal muscles, and it mediates signals from the enteric nervous system<sup>[12-15]</sup>. ICCs were found in the murine<sup>[16]</sup>, *Homo sapiens*<sup>[17]</sup> and guinea pig gallbladders<sup>[18]</sup>. Both the CCK-AR and CCK-BR mediate the contraction of the guinea pig ileum<sup>[19-21]</sup>, whereas the guinea pig gallbladder contraction is mediated solely by CCK-AR<sup>[22]</sup>. It is considered that CCK-induced gallbladder contractions are controlled through CCK-ARs both on the vagal nerve in stimulating endogenous release of acetylcholine and on the gallbladder to directly stimulate muscle contraction in dogs<sup>[23]</sup>. The recent availability of antibodies directed against the CCK-AR has made it possible to identify the receptor by immunocytochemistry<sup>[24]</sup> and immunohistochemistry<sup>[25]</sup>. It has been reported that some ICCs in the rat pylorus express CCK-ARs<sup>[26]</sup>. Therefore, we used an immunohistochemical approach to localize the CCK-AR in the gallbladder. Surprisingly, we found that the interstitial cells of Cajal express CCKARs to a high degree. We also measured the effect of atropine,

tetrodotoxin and CCK on the gallbladder muscle strips, and the contractions of the muscle strips with ICC removed in the guinea pig. This is the first study in which the CCK-induced gallbladder contraction through CCK-AR on the gallbladder ICC was investigated using the guinea pig as the experimental animal.

## MATERIALS AND METHODS

### Animals

Adult guinea-pigs (250-300 g) were obtained by mail from Animal Experiment Center of Wuhan University. The animal-testing protocol used in the present investigation was approved by the Institutional Animal Care and the Use Committee of the Wuhan University.

### Chemicals

All the chemicals and bovine serum albumin were obtained from Sigma. The rat monoclonal antibodies against C-kit were purchased from R&D. The Rabbit anti-CCK-AR polyclonal antibody was purchased from Chemicon. M199 were purchased from Sigma. All the secondary antibodies were bought from Beijing Zhongshan Biotechnology CO., LTD.

### Experimental procedures

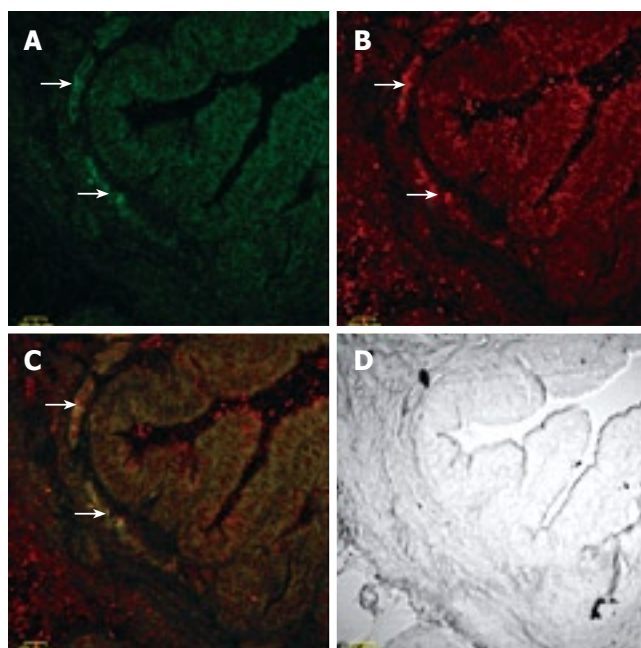
**Demonstration of CCK-AR on the gallbladder tissue by immunohistofluorescence:** The gallbladder was fixed with 4% paraformaldehyde at 4°C for 16 h. Following fixation, the tissues were embedded and cut for immunohistochemistry. Next, they were washed and incubated with normal bovine serum albumin for 10 min. They were subsequently incubated with rat anti-kit monoclonal antibody, with rabbit anti-CCK-AR polyclonal antibody added to the anti-kit solution at 4°C for 24 h. The sections were incubated with the secondary antibody after the first antibody. CCK-AR polyclonal antibody was visualized using the goat anti-rabbit IgG conjugated to Cy3 and the C-kit was visualized using the rabbit anti-rat antibody conjugated to FITC for 2 h at room temperature. A negative control without primary antibody was performed.

**Demonstration of CCK-AR on the gallbladder ICCs by immunohistofluorescence:** The surrounding tissue of guinea pig's gallbladder was cleaned off and cut longitudinally. The tissue was pinned to the base of a dish filled with Sylgard elastomer with the mucosal side facing up. The mucosa was removed by sharp dissection, and the muscular layer tissues were cut out and incubated at 37°C for 23 min in a Ca<sup>2+</sup>-free physiological saline solution (PSS mmol/L: NaCl 135.0, KCl 5.0, glucose 10.0, Hepes 10.0, and MgCl<sub>2</sub> 1.2; pH set to 7.4 with NaOH) containing type II collagenase (1.2 mg/mL), soybean trypsin inhibitor (2.0 mg/mL) and bovine serum albumin (BSA; 2.0 mg/mL). The pieces of tissue were then rinsed five times with Ca<sup>2+</sup>-free PSS and gently triturated with a wide-bore glass pipette to yield the isolated cells. Aliquots of the suspension of the cells were then placed in glass coverslips, and they were left at 4°C for 45-60 min to attach onto the bottom of coverslips for culturing. Cells were washed

four times with Krebs-Ringer buffer (KRB mmol/L: NaCl 118.5, KCl 4.5, MgCl<sub>2</sub> 1.2, NaHCO<sub>3</sub> 23.8, KH<sub>2</sub>PO<sub>4</sub> 1.2, dextrose 11.0 and CaCl<sub>2</sub> 2.4; pH at 7.4) containing penicillin (200 U/mL), streptomycin (200 mg/mL) and amphotericin B (0.5 mg/mL), and they were placed in M199 medium (Sigma) containing similar concentrations of antibiotics and antimycotic. The cells were incubated at 37°C (90% humidity and 95% O<sub>2</sub>-5% CO<sub>2</sub>) for 1 d. Cells were fixed with 4% paraformaldehyde at 4°C for 16 h, and then it was washed and incubated with normal bovine serum albumin for 10 min. Tissues were processed with protocol identical to the cells. The cells and tissues were examined under the TCS SP2 MP laser scanning confocal microscope (Leica, Germany). With an excitation beam (488 nm) produced by argon, the emitted fluorescence was captured using Leica Confocal software running on Windows XP work station.

### Contractile response of the gallbladder muscle strip to CCK-8:

(1) CCK-Induced Contractions on Common Muscle Strips. The adult guinea-pigs sent in were killed by cervical dislocation. The gallbladder was removed and opened, and it was washed several times in Krebs solution to remove bile. Four strips of each gallbladder measuring approximately 0.2 cm × 0.8 cm were mounted in organ baths containing Krebs solution (30 mL) with the following composition (mmol/L): NaCl 120.35, KCl 5.9, MgCl<sub>2</sub> 2.5, NaH<sub>2</sub>PO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 15.5, CaCl<sub>2</sub> 2.5, and glucose 11.5; pH 7.4. The organ bath was maintained at 37°C and gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. A resting 1 g pre-load was applied to each muscle strip and was allowed to equilibrate for 1 h, and during which time the Krebs solution was changed every 20 min. During the equilibration period, the tension was adjusted to 1 g when required. Mechanical activity was recorded using isometric transducers (World Precision Instruments, Stevenage, Herts, U.K.). Tension was continuously monitored and recorded using a MacLab data acquisition system (AD Instruments Ltd., Hastings, U.K.). A dose-response curve for CCK-8 was constructed by adding CCK-8 to the medium in a cumulative way, and the effect of pretreatment with 10<sup>-6</sup> mol/L atropine, 3 × 10<sup>-7</sup> mol/L tetrodotoxin (ITX) on CCK-8 induced gallbladder contraction was also examined. (2) CCK-Induced Contractions on Muscle Strips with ICCs Removed. Methylene blue staining is a nonspecific but effective and time-dependent staining method for ICCs. After the removal of the mucosa, muscle strips were washed with fresh oxygenated Krebs solution (in mmol/L: NaCl 120.35, KCl 5.9, MgCl<sub>2</sub> 2.5, NaH<sub>2</sub>PO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 15.5, CaCl<sub>2</sub> 2.5, and glucose 11.5; pH at 7.4) and then incubated in Krebs solution containing 50 μmol/L methylene blue at 37°C bubbled with 95% O<sub>2</sub>-5% CO<sub>2</sub> (v/v) for 40 min in the dark with intermittent visual checking of the staining intensity. After staining, the muscle strips were immediately put under the light (50 mW/cm<sup>2</sup>) for 5 min. Next, CCK-induced contractions on muscle strips with ICCs removed were done as mentioned above for the common muscle strips. Results were expressed as mean ± SE. (3) Transmission Electron Microscopy. We used transmission electron microscopy to identify the effect of methylene blue



**Figure 1** Overview of CCKAR-immunoreactive ICC seen on cross section of guinea pig gallbladder. **A:** Low-magnification montage showing C-kit positive cells (arrow) distributed over a band of gallbladder muscle adjacent to the mucosa; **B:** Low-power montage showing CCKAR-immunoreactive cells distribute mainly in the muscular layers of guinea pig gallbladder; **C:** Compound micrographs from (A) and (B). Some, but not all C-kit immunoreactive (green) ICC colocalize CCKAR (red), meanwhile, not all CCKAR-immunoreactive cells are ICC; **D:** Phase-contrast micrographs of the same gallbladders wall at the same magnification.

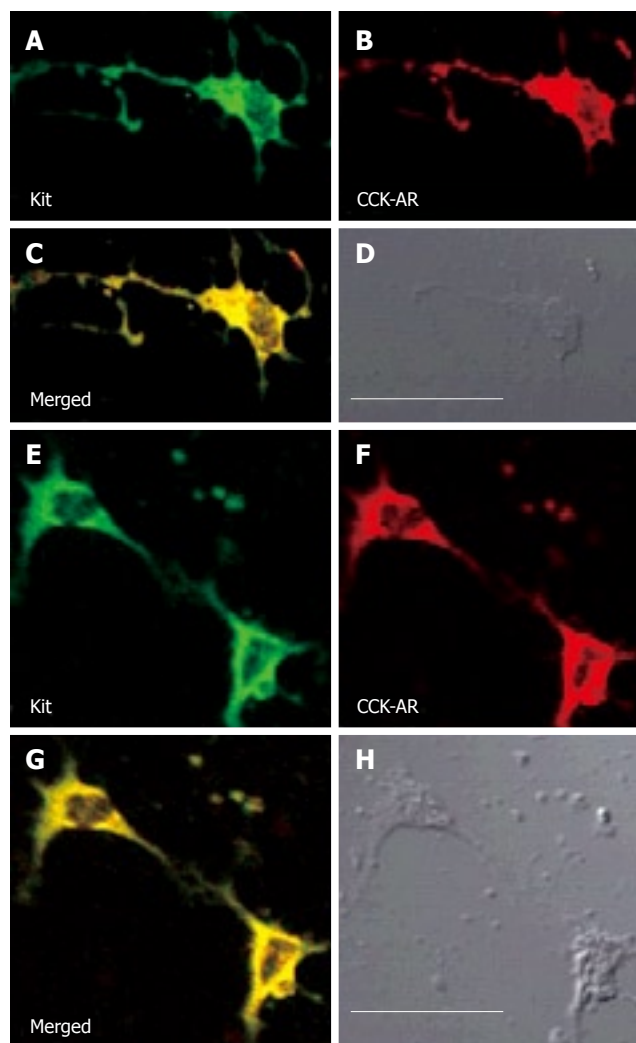
with light on gallbladder ICC. The muscle strips removed ICC were fixed in 2.5% GA in 0.1 mol/L (PB) for 2 h or overnight. They were then washed in 0.1 mol/L PB ( $2 \times 15$  min) and post-fixed with 2% osmium tetroxide in 0.1 mol/L PB (2 h). After another wash with 0.1 mol/L PB ( $2 \times 15$  min), they were dehydrated through ascending grades of ethanol (50, 75, 95 and  $2 \times 100\%$ , 15-20 min each) and embedded in an epoxy resin, Quetol-812. Ultra-thin sections were cut using a Reichert OMU4 ultramicrotome with a diamond-cutting knife. Sections were picked up on 200 mesh copper grids and stained with 2% uranyl acetate in 30% ethanol, and this was followed by Sato's lead staining. Grids with sections were viewed under the OPTON EM10C transmission electron microscope.

## RESULTS

### *Demonstration of CCK-AR on the gallbladder*

**Tissue sections immunohistochemistry:** In cross sections of the gallbladder, intensely CCK-AR immunoreactive interstitial cells were found mainly in the muscular layers. Labeling in the most intensely stained cells was already optimal at an antibody concentration of 1:8000. At low antibody concentrations, there were very little staining found on smooth muscle cells of the gallbladder muscle, yet, at higher concentrations, the gallbladder muscle showed considerable immunoreactivity, particularly in its inner portions (Figure 1).

**Cultured cells immunohistochemistry:** CCK-AR



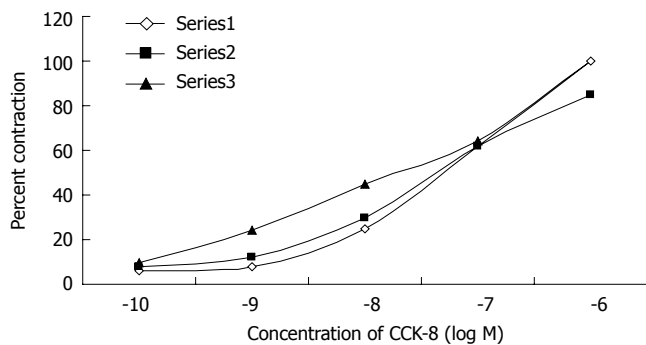
**Figure 2** Colocalization of CCKAR immunoreactivity with markers for ICC, kit in guinea pig gallbladder cultured ICC. CCK-AR immunoreactive ICC in the muscle of the gallbladder is distinctive of their labeled processes. ICC possessed several processes (**A-D**); C-kit immunoreactive (green) ICC colocalize CCKAR (red); **C:** Compound micrographs from (A) and (B); **D:** Phase-contrast micrographs of the same gallbladder ICC at the same magnification; Often two or more labeled ICC were aggregated into a network (**E-H**), making it difficult to discern the individual cells; C-kit immunoreactive (green) ICCs colocalize CCKAR (red); **G:** Compound micrographs from (E) and (F); **H:** Phase-contrast micrographs of the same gallbladder ICCs at the same magnification; All these ICCs were big and had a large and angular nucleus; Bar (in **D**) 30  $\mu$ m for (**A-D**) and (in **H**) 30  $\mu$ m for (**E-H**).

immunoreactive ICC in the muscle of the gallbladder differed in the complexity and shape of their labeling processes (Figure 2). In one extreme end, the processes showed multiplicity and the results were frequently subdivided into fine filaments and peaks (Figure 2A-D). In another end, ICCs possessed only three smooth processes and two labeled ICC were aggregated into the network (Figure 2E-H).

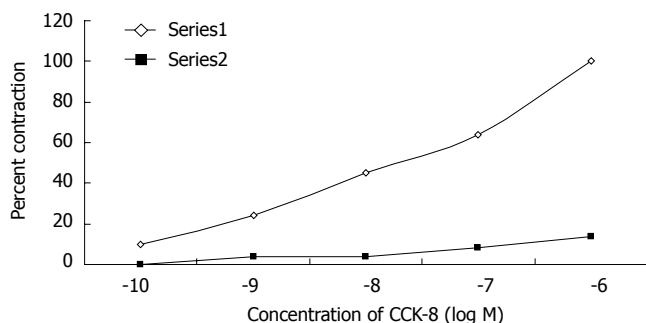
### *CCK-8-induced contractions on gallbladder muscle strips*

The contractile responses of muscle strip to CCK-8 was observed at  $10^{-10}$  mol/L, and they reached the maximum at  $10^{-6}$  mol/L as shown in Figures 3 and 4. CCK-8 stimulated tonic contraction in a concentration-dependent manner. The dose response curve for CCK-8 was not affected





**Figure 3** Effect of atropine and TTX on CCK-8 induced contraction of gallbladder muscle strips. The addition of atropine ( $10^{-6}$  mol/L) or TTX ( $3 \times 10^{-7}$  mol/L) to the medium had no effect on the control dose-response curve for CCK-8. Each value is the mean  $\pm$  SE obtained in 5 observations and observed at  $10^{-10}$ ,  $10^{-9}$ ,  $10^{-8}$ ,  $10^{-7}$ ,  $10^{-6}$  mol/L. Series 1: CCK-8+TTX; Series 2: CCK-8+atropine; Series 3: CCK-8 alone.



**Figure 4** Effects of CCK-8 on contraction of gallbladder muscle strips *in vitro*. Removing the ICC of the gallbladder significantly shifted the control dose-response curve to the right. Each value is the mean  $\pm$  SE obtained in 5 observations and observed at  $10^{-10}$ ,  $10^{-9}$ ,  $10^{-8}$ ,  $10^{-7}$ ,  $10^{-6}$  mol/L. Series 1: Common gallbladder muscle strip; Series 2: Gallbladder muscle strip removed ICC.

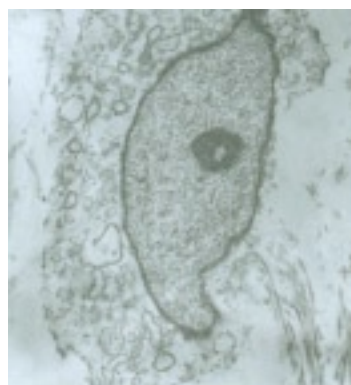
by pretreatment of the muscle strips with atropine ( $10^{-6}$  mol/L) or TTX ( $3 \times 10^{-7}$  mol/L) (Figure 3). The purpose of using atropine and TTX is to eliminate the effect of nervous system on the contractions of the guinea pig gallbladder. When we removed the ICCs from the gallbladder muscle strip, it significantly shifted the dose response curve to the right (Figure 4).

**Transmission electron microscopy**

Methylene blue with light destroyed the structure of the gallbladder ICCs. The ICCs were swollen, and they showed low contrast for the cytoplasm. Cells became smaller and processes diminished. All these changes result in the reduction of contractile function of the gallbladder (Figure 5).

**DISCUSSION**

In this study, we demonstrated that: (1) CCK-AR are present on the gallbladder interstitial cells of Cajal; (2) The gallbladder contractions evoked by CCK-8 through the CCK-AR on the gallbladder ICC. This is the first report on on ICC of guinea pig *in vitro* studies on the mechanism of gallbladder contraction evoked by CCK-8 through CCK-AR.



**Figure 5** The effect of methylene blue with light on gallbladder ICC. After incubation with methylene and light, ICC on gallbladder was swollen and had low cytoplasmic, and the nucleolus became smaller and processes diminished.

The study has clearly shown that CCK-AR are present on gallbladder ICCs by *in vitro* study with immunohistochemistry, immunocytochemistry and muscle strip trials. The CCK receptors on the gallbladder have been demonstrated previously by autoradiography in the guinea pig. In this study, we have clearly shown that CCK-AR exists in the gallbladder smooth muscle layer in the guinea pig, and to the best of our knowledge, the CCK-AR has not yet been demonstrated by any other studies. When we initially used high primary antibody concentrations, the entire gallbladder exhibited immunoreactivity. With lower antibody concentration of 1:8000, there were little immunoreactivity associated with the smooth muscle, but intense staining of ICC in the gallbladder sections. CCK may bind to CCK-ARs on both smooth muscle and ICC.

We demonstrate here that a population of guinea pig gallbladder ICC strongly expresses CCK-AR immunoreactivity. Identification of ICC was based on their typical morphological traits such as clusters of mitochondria, well-developed smooth endoplasmic reticulum, dense body, lysosomes, distinctive caveolae and abundant fibers around the lysosomes. In addition, co-localization of C-kit double labeling with CCK-AR showed that only a subpopulation was localized mainly to a contiguous band of circular muscle of the guinea pig.

In the gallbladder, ICCs intimately contact with smooth muscle cells with gap junction, and they are responsible for the generation of smooth muscle rhythmic activity<sup>[18]</sup>. The finding that gallbladder ICCs strongly express CCK-AR suggests a CCK-induced gallbladder activity. ICCs may mediate some of the effect of CCK on gallbladder pressure. It was previously shown that the gallbladder muscle strip contractions evoked by CCK are mediated by a direct myogenic action<sup>[27,28]</sup> and inhibited by a specific CCK-AR antagonist in some species<sup>[29]</sup>. The next question is how CCK action is mediated to the gallbladder through the CCK-AR on the ICC. When we removed the ICCs in the gallbladder, the CCK-8 induced contraction dose response curve shifted significantly to the right. Liu<sup>[30]</sup> showed that intense illumination after incubation with methylene blue resulted in specific lesions of dogs' ICCs, and it was verified by electron microscopy. The ICCs were swollen and had low cytoplasmic contrast. They had barely discernible mitochondria with few caveolae, and the plasma membranes was punctuated so that they were presumably ruptured. All changes in ICCs result in the abolishing of the slow wave. The effects of methylene

blue with light in ICC were specific only to ICCs, but not the associated smooth muscle cells. In our study, the contractile ability of the guinea pig gallbladder muscle strips without the evocation of CCK-8 was sharply weakened. This phenomenon implied that CCK-8 evoked the contraction of the gallbladder through the gallbladder ICC.

Immunohistofluorescence and muscle strip trials proved that CCK acted not only on the CCK-AR on the smooth muscle, but also on the CCK-AR on the gallbladder ICC. This may be the first indication that ICC do not only generate slow-wave pacemaker activity and mediate effect of enteric neurotransmitters, but also mediate the effects of circulating hormones on smooth muscle activity.

In conclusion, we have shown that the CCK-evoked contractions are mediated through direct action on CCK-AR on the gallbladder ICC.

## COMMENTS

### Background

It has been convinced that the interstitial cells of Cajal (ICC) in rat gastric pylorus expresses CCK-ARs to a high degree. The fact that some ICC in the rat pylorus expresses CCK-ARs suggests the possibility that circulating CCK can influence gastrointestinal motility via ICC. It is well known that CCK regulates the guinea pig gallbladder contraction by CCK-AR. The ICC were also found in the murine, human and guinea pig gallbladder. It deserved us to study that if there are CCK-ARs on the guinea pig gallbladder ICC and how CCK regulates gallbladder contraction through CCK-AR on ICC.

### Research frontiers

ICC does not only generate slow-wave pacemaker activity and mediate effects of enteric neurotransmitters, but also mediate effects of circulating hormones such as CCK on smooth muscle activity.

### Innovations and breakthroughs

In the present study, it was demonstrated that: (1) CCK-AR is present on the gallbladder interstitial cells of Cajal; (2) The gallbladder contractions evoked by CCK-8 through the CCK-AR on the gallbladder ICC. This is the first report on guinea pig *in vitro* studies on the mechanism of gallbladder contraction evoked by CCK-8 through CCK-AR on ICC.

### Applications

It has been shown that CCK evoked contraction is mediated through direct action on CCK-AR on the gallbladder ICC. This maybe some use of study in gallbladder motility disorders, such as cholelithiasis.

### Peer review

This manuscript demonstrates CCK-AR receptor immunoreactivity in the gallbladder of the guinea pig. Furthermore, the authors demonstrate that administration of CCK8 to gallbladder muscle strips caused muscle contraction. This study is of important clinical significance and should be of interest to the readers.

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