Investigation of cholecystokinin receptors in the human lower esophageal sphincter

Jun-Feng Liu, Jian Zhang, Xin-Bo Liu, Paul A Drew

AIM: To compare the binding of cholecystokinin (CCK)-8 to CCK receptors in sling and clasp fibers of the human lower esophageal sphincter.

METHODS: Esophageal sling and clasp fibers were isolated from eight esophagectomy specimens, resected for squamous cell carcinoma in the upper two thirds of the esophagus, which had been maintained in oxygenated Kreb's solution. Western blot was used to measure CCK-A and CCK-B receptor subtypes in the two muscles. A radioligand binding assay was used to determine the binding parameters of \(^3\)H-CCK-8S to the CCK receptor subtypes. The specificity of binding was determined by the addition of proglumide, which blocks the binding of CCK to both receptor subtypes.

RESULTS: There was no significant difference between the sling and clasp fibers of the human lower esophageal sphincter in the amount of CCK-A (integrated optical density (IOD) value: 22.65 ± 0.642 vs 22.328 ± 1.042, \(P = 0.806\)) or CCK-B receptor protein (IOD value: 13.20 ± 0.423 vs 12.45 ± 0.294, \(P = 0.224\)) as measured by Western blot. The maximum binding of radio-labeled CCK-8S was higher in the sling fibers than in the clasp fibers (595.75 ± 3.231 cpm vs 500.00 ± 10.087 cpm, \(P < 0.001\)) and dissociation constant was lower (\(K_d\): 1.437 ± 0.024 nmol/L vs 1.671 ± 0.024 nmol/L, \(P < 0.001\)). The IC\(_{50}\) of the receptor specific antagonists were lower for the CCK-A receptors than for the CCK-B (\(P < 0.01\)).

CONCLUSION: CCK binding modulates the contractile function of the lower esophageal sphincter through differential binding to the CCK-A receptor on the sling and clasp fibers.

Key words: Cholecystokinins; Cholecystokinins-A receptor; Cholecystokinins-B receptor; Radioligand binding; Lower esophageal sphincter; Sling fibers; Clasp fibers
INTRODUCTION

The lower esophageal sphincter (LES) is the incresatate muscle bundle located at the esophagogastric junction, and includes the sling fibers from the greater curvature and clasp fibers from the lesser curvature of the stomach[8]. The LES can open to allow liquids or solids to enter the stomach, or to permit vomiting or belching. At other times the basal tone of the LES is intended to prevent abnormal reflux of gastric contents into the esophagus[2]. The regulation of the LES is complex, involving interplay between the nervous and hormonal systems, as well as local myogenic influences[14]. In particular, gastrointestinal peptide hormones play important roles in its regulation[5,8].

The cholecystokinin (CCK) are a family of peptide hormones which have important roles in regulating gastrointestinal motility and the delivery of nutrients to the small intestine[5,10]. The individual members are identified by the number of amino acids in the hormone following post-translational modification of the CCK gene product, preprocholecystokinin (e.g., CCK-58, CCK-8). The receptors for CCK are divided into two subtypes, CCK-A and CCK-B, based on their affinities to CCK analogues, gastrin and specific antagonists. Gastrin and CCK are similar in structure, and both bind to CCK-B receptors. We have previously shown that human sling and clasp fibers contract in response to both gastrin and CCK-8, but the sling fibers have stronger contractions than the clasp fibers[9].

Structural and functional abnormalities of the LES may result in esophageal diseases[11,12]. An incompetent LES permits gastro-esophageal reflux, which damages the esophageal epithelial lining and may lead to complications such as esophagitis, Barrett’s esophagus or cancer[13,14]. Currently, antireflux surgery, typically Nissen fundoplication, is the mainstay treatment to prevent reflux, but it is invasive and has a number of potential side-effects. A better understanding of the physiology of the LES may lead to medical interventions to prevent or reduce reflux, avoiding the need for surgery. In this study we investigated if the differential response of sling and clasp fibers to CCK-8 correlates with differential binding characteristics of CCK-8 to the CCK receptors in these fibers.

MATERIALS AND METHODS

Tissues

Specimens of the esophagogastric junction were obtained from 5 men and 3 women (mean age 53 years, range 45-75 years) who underwent esophagectomy for squamous cell carcinoma in the upper two-thirds of the esophagus. Patients with heartburn symptoms or motility disorders, such as achalasia of the esophagus or dermatosclerosis, were excluded from the study. Immediately after removal from the patient the esophagogastric junction tissue was placed in oxygenated Kreb’s solution and transported to the laboratory. Frozen section histology was performed to confirm absence of tumor in the specimens. The sling and clasp fibers (Figure 1) were separated as described previously[10]. The experimental protocol was approved by the Ethics Committee of the Fourth Hospital, Hebei Medical University.

Western blot

Membrane proteins were isolated using the Eukaryotic Membrane Protein Extraction kit (Pierce, Rockford, IL, United States), following the manufacturer’s instructions. The CCK-A and CCK-B receptors in the membrane protein preparation were detected by Western blot as previously described[13]. A goat polyclonal antibody to human CCK-A receptor (1:400 dilution) and a goat polyclonal antibody to human CCK-B receptor (Santa Cruz, United States) were each used at a dilution of 1:400. The secondary antibody was a donkey anti-goat IgG conjugated to peroxidase, used at a 1:2000 dilution and developed with DAB. The integrated optical density (IOD) of each band was determined using the Gel-Pro analyzer software package (Media Cybernetics, United States).

Binding studies

The binding studies were carried out as described by Salvatore et al[10]. The membrane protein isolate was incubated, at a final concentration of 0.15 mg/mL with serial dilutions of 1H-CCK-8S (Sigma, United States), ranging from 6.4 nmol/L to 0.05 nmol/L in a total volume of 200 μL for 10 h at 4 ℃ with shaking every 30 min. The specificity of binding was determined by the addition of 5 μmol/L proglumide, which blocks the binding of CCK to both receptor subtypes. Bound ligand was isolated by filtration under vacuum on Whatman GF/B filters which were then washed three times with ice-cold HEPES buffer (130 mmol/L NaCl, 5 mmol/L MgCl2 and 10 mmol/L HEPES, pH 7.4). Bound radioactivity was measured by liquid scintillation counting (Model LS 6500, Beckman Instruments, Fullerton, CA, United States). Nonspecific binding, defined as the binding of radiolabelled ligand in the presence of 10 nmol/L CCK, was subtracted from the total binding measured in each assay. Competitive inhibition was determined using 7.5
nmol/L $^3$H-CCK-8S with CR1409 as an antagonist to the CCK-A receptor or CR2945 to the CCK-B receptor (Sigma, United States). Data were analyzed and competitive curves constructed from the mean of triplicate measurements. The dissociation constant of the radioligand receptor complex (Kd) and the maximum binding value (Bmax) were calculated using GraphPad Prism version 4.0 (GraphPad Software Inc., San Diego, CA, United States). The inhibitory binding constant Ki was calculated from the IC50 according to the Cheng-Prusoff equation, $K_i = IC_{50}(1 + L/K_d)$, where L is the concentration of the radioligand, IC50 is the concentration of drug causing 50% inhibition of the specific radioligand binding, and Kd is dissociation constant [17].

**Statistical analysis**

Data are expressed as mean ± SD, and groups were compared by the paired Student’s t test using the SPSS statistical program. Differences were considered statistically significant when P < 0.05.

**RESULTS**

**Receptor expression**

Both CCK-A and CCK-B receptors were measured in the membrane protein extract of the sling and the clasp muscle fibers by Western blot (Figure 2). The results in Table 1 show that the IOD for the beta-actin loading control did not differ between the fibers. There were no significant differences between the sling and clasp fibers in the IOD for the CCK-A (t = 0.263, P = 0.806) or the CCK-B (t = 1.439, P = 0.224) receptors.

**Binding studies**

The binding of $^3$H-CCK-8S to the CCK receptor was specific in both the sling and clasp fibers. A typical saturation isotherm plot for the binding of $^3$H-CCK-8S to human cell membrane protein extract is shown in Figure 3. The mean Bmax and Kd values for all eight sling and clasp muscle preparations analysed (clasp fibers: 500.00 ± 10.09 nmol/L vs 1.671 ± 0.024; sling fibers: 595.75 ± 3.23 vs 1.437 ± 0.024; t = 9.040 vs 6.898) differed significantly between the sling and the clasp fibers (each P < 0.001). The results in Figure 4 show the competitive inhibition curves of the specific CCK-A receptor antagonist, CR1409, and the specific CCK-B receptor antagonist, CR2945, for the binding of $^3$H-CCK-8S to the membrane protein extract from the sling and clasp fibers. The mean IC50 values for CR1409 and CR2945 for all eight sling and clasp muscle preparations analysed are shown in Table 2. There were no significant differences between the sling and clasp fibers in the IC50 for CR1409 (t = 1.72, P = 0.161) or CR2945 (t = 1.93, P = 0.126). In both the sling and clasp fibers the IC50 for CR1409 was significantly higher than that for CR2945 (P = 0.001 and P < 0.001 respectively). The pKt values were also greater for CR1409 than for CR2495 in both the sling and clasp fibers (Table 2) (each P < 0.001).

**DISCUSSION**

The gastrointestinal hormone CCK plays an important role in the regulation of gastrointestinal motility. We have previously shown that the sling and clasp fibers, which contribute to the tone of the LES, contract in re-
Table 2  IC\textsubscript{50} values and pKi values

|       | CR\textsubscript{1409}          | CR\textsubscript{2945}          | t     | \(P\) value |
|-------|----------------|----------------|------|-----------|
| Clasp fibers | 3.165 ± 0.187 | 9.583 ± 0.501 | 11.99 | < 0.001   |
| Sling fibers | 2.798 ± 0.104 | 8.147 ± 0.551 | 9.53  | 0.001     |
| P value | 0.161          | 0.126          |      |           |
| Clasp fibers | 8.476 ± 0.065 | 8.018 ± 0.028 | 12.69 | 0.001     |
| Sling fibers | 8.556 ± 0.022 | 8.090 ± 0.042 | 11.73 | 0.001     |
| P value | 0.156          | 0.058          |      |           |

The IC\textsubscript{50} values and pKi values for the CCK-A receptor antagonist CR1409 and the CCK-B receptor antagonist CR2945 in human sling and clasp fibers (\(\times 10^{-9}\) mol/L; mean ± SD).

**Figure 4** Representative competitive inhibition curves of the binding of \(^3\)H-cholecystokinin-8S to membrane proteins isolated from the human sling (A) and clasp (B) fibers. The antagonists used were CR-1409 (selective for cholecystokinin (CCK)-A receptors) and CR-2945 (CCK-B receptors). Binding is expressed as the percentage of radioactivity specifically bound in the absence of antagonists.
et al\[3\] found in cats that CCK induced LES contraction through a preganglionic cholinergic mechanism involving a nicotinic synapse, but induction of relaxation occurred predominantly at a postganglionic site involving adrenergic modulation. Based on these experiments it was proposed that there is animal-to-animal variability in the balance of excitatory and inhibitory mechanisms to the LES, which determines the effect of a mediator which is capable of activating both mechanisms\[35\].

The most likely explanation for the difference in action of CCK described in these reports and our study is that we studied the effect of the hormone on contraction of the isolated sling and clasp fibers in vitro, whereas in the other studies the LES pressure was measured in vivo\[3\]. The location of a high pressure zone at the gastroesophageal junction and the asymmetric distribution of the pressure, as measured by esophageal manometry, are in concordance with the position and arrangement of the two bundles of fibers\[36\]. Thus, while it is clear that the sling and clasp fibers contribute to the generation of the LES pressure, they are not the only muscles involved. Additionally, in vitro studies permit the measurement of effects due to CCK alone, whereas with in vivo studies it is difficult to control for all the variables which may impact on a function as complex as that which regulates LES pressure.

In conclusion, we found no difference in the amount of CCK-A or CCK-B receptors between human sling and clasp fibers, as measured by Western blot, but the clasp fibers bound more radiolabelled CCK-8 than the clasp fibers. Our binding and inhibition data are consistent with the CCK-A receptor playing an important role in mediating the contractile function of the LES, acting through differential effects on the sling and clasp fibers.

**COMMENTS**

**Background**

The sling fibers of the human lower esophageal sphincter contract more strongly in response to gastrin and CCK-8 than the clasp fibers. Authors investigate a possible explanation that the binding of CCK-8 to CCK receptor subtypes differs between these fibers.

**Research frontiers**

It is well known that the muscles comprising the lower esophageal sphincter have specific structural and physiological characteristics. The regulation of the lower esophageal sphincter is complex, involving an interplay between the nervous and hormonal systems, as well as local myogenic influences. In particular, gastrointestinal peptide hormones play an important role in the regulation of the esophagus, lower esophageal sphincter and the stomach. CCK antagonists may have therapeutic value in the prevention or treatment of gastro-esophageal reflux disease including esophagitis, Barrett’s esophagus and esophageal adenocarcinoma.

**Peer review**

The results are interesting and suggest that CCK binding modulates the contractile function of the lower esophageal sphincter through differential binding to the CCK-A receptor on the sling and clasp fibers.

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