Cholecystokinin octapeptide increases free intracellular calcium of guinea pig cardiomyocytes through activation of Ca\(^{2+}\) channel and tyrosine kinase

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Abstract: The aim of the present study was to explore the effect of cholecystokinin octapeptide (CCK-8) on [Ca\(^{2+}\)]\(_i\), and its signal transduction mechanism in isolated guinea pig cardiomyocytes. [Ca\(^{2+}\)]\(_i\) was measured by laser scanning confocal microscopy in single ventricular myocytes which were dissociated by enzymatic dissociation method and loaded with Fluo 3-AM. The changes in [Ca\(^{2+}\)]\(_i\) were represented by fluorescent intensity (F) or relative fluorescent intensity (F/F\(_0\))\%. The results obtained are as follows. (1) In the normal Tyrode's solution containing 1.0 mmol/L Ca\(^{2+}\), CCK-8 (1~10\(^4\) pmol/L) elicited a rapid and marked increase in [Ca\(^{2+}\)]\(_i\). (2) When cardiomyocytes were pretreated with the Ca\(^{2+}\) chelator EGTA (3 mmol/L) and Ca\(^{2+}\) channel antagonist nisoldipine (0.5 µmol/L) for 5 min, CCK-8 (10\(^6\) pmol/L) caused a slow and small increase in [Ca\(^{2+}\)]\(_i\) (P < 0.01). (3) Pretreatment with the nonselective CCK receptor (CCK-R) antagonist proglumide (6 µmol/L) for 5 min could inhibit the increase of [Ca\(^{2+}\)]\(_i\), induced by CCK-8 (10\(^6\) pmol/L) (P < 0.01). The results suggest that CCK-8 increases the [Ca\(^{2+}\)]\(_i\) via activating the receptor-operated Ca\(^{2+}\) channel and eliciting the influx of Ca\(^{2+}\) in isolated guinea pig cardiomyocytes, in which tyrosine kinase may be involved.

Key words: cholecystokinin; cardiomyocyte; fluorescent intensity; Ca\(^{2+}\) channel; confocal microscopy; receptor

八肽胆囊收缩素激活钙离子通道和酪氨酸激酶诱导豚鼠心肌细胞内的游离钙增高

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摘要：探讨八肽胆囊收缩素 (CCK-8) 对豚鼠单个心肌细胞内游离钙浓度 ([Ca\(^{2+}\)]\(_i\)) 的影响及其信号转导机制。Fluo 3-AM 标记酶消化法分离的单个心室肌细胞，用激光共聚焦显微镜测定细胞内 [Ca\(^{2+}\)]\(_i\) 的浓度。[Ca\(^{2+}\)]\(_i\) 的变化用荧光强度 (F) 和相对荧光强度 (F/F\(_0\))\% 表示。实验结果如下：(1) 在含 Ca\(^{2+}\) 1.0 mmol/L 的Tyrode’s液中，CCK-8 (1~10\(^6\) pmol/L) 均可引起 [Ca\(^{2+}\)]\(_i\) 快速显著上升 (P < 0.01)。 (2) 用钙离子螯合剂 EGTA (3 mmol/L) 和钙离子阻断剂 nisoldipine (0.5 µmol/L) 诱导心肌细胞5 min, CCK-8 (10\(^6\) pmol/L) 仅可引起 [Ca\(^{2+}\)]\(_i\) 缓慢轻度上升 (P > 0.01)。 (3) 用非选择性 CCK 受体拮抗剂 proglumide (6 µmol/L) 或酪氨酸激酶抑制剂 genistein (1 µmol/L) 诱导心肌细胞5 min, 则完全抑制 CCK-8 诱导的 [Ca\(^{2+}\)]\(_i\) 升高 (P < 0.01)。 CCK-8 可通过激活其受体控制的 Ca\(^{2+}\) 通道，引起 Ca\(^{2+}\) 内流，诱导细胞内 Ca\(^{2+}\) 释放，引起豚鼠单个心肌细胞内 [Ca\(^{2+}\)]\(_i\) 上升，此作用可能由酪氨酸激酶介导。

关键词：八肽胆囊收缩素; 心肌细胞; 荧光强度; 钙通道; 共聚焦显微镜; 受体
Cholecystokinin (CCK) functions as a neurotransmitter in the central nervous system and gastrointestinal tract as well as a hormone in the gastrointestinal tract, and has numerous biologic effects[1]. Cholecystokinin octapeptide (CCK-8) is a key biological fragment of endogenous CCK. Previous studies showed that CCK-8 elicited dose-dependent increase in arterial blood pressure[2,3], an effect that was inhibited by CCK-A receptor (CCK-AR) antagonist[2,3]. Our recent study indicated that CCK-8 could enhance cardiac function in a dose-dependent manner through activation of CCK-receptor (CCK-R) on myocardium[3]. It is well established that the increase in [Ca$^{2+}$] is the key factor that augments the strength of myocardial contraction. However, the effects of CCK-8 on [Ca$^{2+}$] level in cardiomyocytes remain unclear. The present study was undertaken to observe the effects of CCK-8 on [Ca$^{2+}$] level and the signal transduction mechanism involved.

1 MATERIALS AND METHODS

1.1 Preparation of guinea pig cardiomyocytes. Six male guinea pigs (300–500 g) were provided by the Experimental Animal Center of Hebei Province (grade II, Certificate No 04064). Animals were first injected intraperitoneally with 1000 U of heparin and were decapitated 15 min later. Hearts were removed quickly by thoracotomy and rinsed in oxygenated ice-cold Ca$^{2+}$-free Tyrode's solution. Single ventricular myocytes were isolated as Rubin described[6]. Briefly, guinea pig hearts were perfused retrogradely through the aorta at a rate of 9 ml/min with Ca$^{2+}$-free Tyrode's solution for 5 min, and then with the same solution containing collagenase II 360 mg/L, bovine serum albumin (BSA) 160 mg/L, and CaCl$_2$ 34 µmol/L for 10 min at 37°C. The ventricles were cut, and then minced, incubated for 10 min in Ca$^{2+}$-free Tyrode's solution containing 0.1% BSA. Myocytes were harvested after filtration through a nylon mesh, and resuspended in Tyrode's solutions containing different concentrations of Ca$^{2+}$. The concentration of Ca$^{2+}$ was gradually increased to 1 mmol/L.

1.2 Fluo 3-AM loading. Isolated ventricular myocytes were incubated with Fluo 3-AM working solution containing 0.03 % Pluronic F-127 (the final concentration of Fluo 3-AM is 20 µmol/L) at 25°C for 60 min. After incubation, the cells were washed with Ca$^{2+}$-free Tyrode's solutions three times to remove the extracellular Fluo3-AM at 25°C.

1.3 Calcium measurement. The cells were placed into a small pool of Teflon printed slice (from Electromicroscopy Sciences), and covered by cover glass. Only the cells with rod shaped and visible striations were used for experiments. The fluorescence signal was detected with confocal laser scanning system (BioRad MR/A2), which was equipped with a Nikon E-600 eclipse microscope. An argon laser was used to excite Fluo-3 at 488 nm and emit at 530 nm. [Ca$^{2+}$] changes were represented with fluorescent intensity ($F_{i}$) or relative fluorescent intensity ($F_{i}/F_{0}$%). $F_{i}/F_{0}$% is the ratio of change in $F_{i}$ to the fluorescence under quiescent condition ($F_{0}$). The images were collected with computer and then processed with confocal assistant and photoshop software. Therefore, the distribution of [Ca$^{2+}$] was recorded both in the quiescent condition and after application of CCK-8 or other drugs.

1.4 Experimental protocols. The experiments were consisted of 3 groups (n=6): (1) effect of CCK-8 on [Ca$^{2+}$]: $F_{i}$ was measured after adding CCK-8 (1–10$^{4}$ pmol/L) to the normal Tyrode's solution containing 1.0 mmol/L Ca$^{2+}$; (2) effect of EGTA or nisoldipine on [Ca$^{2+}$], elevation induced by CCK-8: the preparation was pretreated with the Ca$^{2+}$-chelator EGTA[7] (3 mmol/L) or the calcium channel blocker nisoldipine[8] (0.5 µmol/L) for 5 min, and $F_{i}$ was measured after adding CCK-8 (10$^{4}$ pmol/L); (3) effect of proglumide and genistein on [Ca$^{2+}$], elevation induced by CCK-8: the preparation was pretreated with the nonselective CCK-R antagonist proglumide (6 µmol/L) or tyrosine kinase inhibitor genistein (1 µmol/L) for 5 min, and $F_{i}$ was measured after adding CCK-8 (10$^{5}$ pmol/L).

1.5 Chemicals and solution. Sulfated CCK-8, collagenase type II, taurine, MOPS, HEPES, EGTA, nisoldipine, proglumide and genistein were purchased from Sigma Co. The Ca$^{2+}$-free Tyrode's solution contained NaCl 100, KCl 10, MgSO$_4$ 5, NaH$_2$PO$_4$ 1.2, glucose 20, taurine 10, MOPS 10 mmol/L, pH was adjusted to 7.4 with KOH. The normal Tyrode's solution was used by adding 1.0 mmol/L CaCl$_2$ to the Ca$^{2+}$-free Tyrode's solution.

1.6 Statistic analysis. All data were expressed as means±SD, and analyzed by one-way ANOVA followed by the paired t test for within-group comparison and the unpaired t test for between group comparison. $P$ values less than 0.05 were considered statistically significant.

2 RESULTS

2.1 Effect of CCK-8 on [Ca$^{2+}$] in cardiomyocytes

CCK-8 (1–10$^4$ pmol/L) elicited a rapid and marked
increase in \([\text{Ca}^{2+}]\), in isolated cardiomyocytes in normal Tyrode's solution containing 1.0 mmol/L \(\text{Ca}^{2+}\) \((P<0.01)\). The \([\text{Ca}^{2+}]\), reached the peak level at 30 s, and then restored to baseline after 200 s. CCK-8 \((10^2\ \text{pmol/L})\) produced a marked increase in \([\text{Ca}^{2+}]\) \((P<0.01)\) (Figs. 1A and 2).

2.2 Effect of EGTA and nisoldipine on \([\text{Ca}^{2+}]\) elevation induced by CCK-8 in cardiomyocytes

CCK-8 \((10^2\ \text{pmol/L})\) induced a small and slow increase in \([\text{Ca}^{2+}]\), in cardiomyocytes after pretreatment with EGTA \((3\ \text{mmol/L})\) and nisoldipine \((0.5\ \mu\text{mol/L})\) for 5 min. The increase in \([\text{Ca}^{2+}]\), was inhibited by administration of either EGTA or nisoldipine \((P<0.01)\). The \([\text{Ca}^{2+}]\), reached the peak level at 30–60 s, and then restored to baseline after 120–150 s (Figs. 1B and 3, Table 1).

2.3 Effect of proglumide and genistein on \([\text{Ca}^{2+}]\) elevation in cardiomyocytes induced by CCK-8

Pretreatment with the non-selective CCK-R antagonist proglumide \((6\ \mu\text{mol/L})\) or the tyrosine kinase inhibitor genistein \((1\ \mu\text{mol/L})\) for 5 min inhibited the increase in \([\text{Ca}^{2+}]\), induced by CCK-8 \((10^2\ \text{pmol/L})\) in isolated cardi-
omyocytes ($P<0.01$). The $[\text{Ca}^{2+}]_\text{i}$ fell to values below quiescent condition after 150 s (Fig. 4, Table 1).

### 3 DISCUSSION

Intracellular calcium is an important second messenger and mediates a series of biological actions. It is well known that the increase of $[\text{Ca}^{2+}]_\text{i}$ in cardiomyocyte is the key factor for modulating the strength of myocardial contraction. It is reported that CCK-8 elicited a dose-dependent increase in arterial blood pressure in both conscious and pithed rats\cite{2-4}. Our laboratory has shown that CCK-8 enhances cardiac function and mean arterial pressure (MAP) in a dose-dependent manner in anesthetized rats\cite{5}. However, the roles of $\text{Ca}^{2+}$ in the effects of CCK-8 on cardiac function were unknown. In the present study, we measured directly the effect of CCK-8 on $[\text{Ca}^{2+}]_\text{i}$ in isolated single cardiomyocyte with confocal microscopy, which is a sensitive method to detect the low level fluorescence and has almost no deleterious effect on living cells. Our results showed that CCK-8 ($1\sim10^4$ pmol/L) significantly increased the $[\text{Ca}^{2+}]_\text{i}$ in isolated cardiomyocytes, which could provide a further explanation for the effects of CCK-8 on cardiac function.

$[\text{Ca}^{2+}]_\text{i}$ stemmed from the influx of $\text{Ca}^{2+}$ through the $\text{Ca}^{2+}$ channels and/or triggered the release of $\text{Ca}^{2+}$ from the sarcoplasmic reticulum (SR) via the $\text{Ca}^{2+}$-induced $\text{Ca}^{2+}$ release (CICR) mechanism. Since calcium entry through the $\text{Ca}^{2+}$ channel plays an important part in excitation-contraction coupling, it was thought that the $\text{Ca}^{2+}$ current ($I_{\text{Ca}}$) might be enhanced by CCK-8. The present study demonstrated that CCK-8 only caused a slight and slow increase in $[\text{Ca}^{2+}]_\text{i}$ after pretreatment with EGTA or nisoldipine in isolated cardiomyocytes, suggesting that CCK-8 is able to activate the $\text{Ca}^{2+}$ channels and elicit $\text{Ca}^{2+}$ influx, in turn leads to intracellular $\text{Ca}^{2+}$ increase or directly triggers the release of $\text{Ca}^{2+}$ from the SR, while the latter effect was weaker than the former.

Our recent study showed that CCK-A/BR mRNA expression was identified by RT-PCR in myocardium of rats\cite{5}. Yule reported that stimulation with CCK-8 resulted in an increase in polyphosphoinositides (PPI) hydrolysis and $[\text{Ca}^{2+}]_\text{i}$ in the CHO cell line when the CCK-AR was transfected\cite{9}. Furthermore, studies in rat pancreatic acini suggested that CCK-8 ($10^3$ pmol/L) rapidly stimulated tyrosine phosphorylation by activation of both high and low affinity CCK-AR and produced an increase in $[\text{Ca}^{2+}]_\text{i}$\cite{10}. Similarly, Lutz et al. reported that CCK as a IP$_3$/Ca$^{2+}$-mediated agonist could induce tyrosine phosphorylation of regulatory

### Table 1. Effect of CCK-8 ($10^2$ pmol/L) on $[\text{Ca}^{2+}]_\text{i}$ changes in single isolated cardiomyocytes pretreated with EGTA, nisoldipine, proglumide or genistein

<table>
<thead>
<tr>
<th>Group</th>
<th>$n$</th>
<th>$F_i/F_0$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quiescent condition</td>
<td>6</td>
<td>100.02 ± 1.17</td>
</tr>
<tr>
<td>CCK-8</td>
<td>6</td>
<td>245.93 ± 10.24*</td>
</tr>
<tr>
<td>EGTA+CCK-8</td>
<td>6</td>
<td>136.99 ± 4.88**</td>
</tr>
<tr>
<td>Nisoldipine+CCK-8</td>
<td>6</td>
<td>121.68 ± 7.03**</td>
</tr>
<tr>
<td>Proglumide+CCK-8</td>
<td>6</td>
<td>102.86 ± 6.78*</td>
</tr>
<tr>
<td>Genistein+CCK-8</td>
<td>6</td>
<td>109.63 ± 3.51*</td>
</tr>
</tbody>
</table>

$[\text{Ca}^{2+}]_\text{i}$ changes are represented by $F_i/F_0$. Mean ± SD. *$P<0.01$ vs quiescent condition; **$P<0.01$ vs CCK-8 ($10^2$ pmol/L) in normal Tyrode’s solution; *$P<0.01$ vs pretreatment with proglumide.

Fig. 3. Effect of EGTA and nisoldipine on $[\text{Ca}^{2+}]_\text{i}$ elevation induced by CCK-8 ($10^2$ pmol/L) in single isolated cardiomyocytes. $[\text{Ca}^{2+}]_\text{i}$ changes were represented by relative fluorescent intensity ($F_i/F_0$%). †, addition of CCK-8 ($10^2$ pmol/L).

Fig. 4. Effect of proglumide and genistein on $[\text{Ca}^{2+}]_\text{i}$ elevation induced by CCK-8 ($10^2$ pmol/L) in single isolated cardiomyocytes. $[\text{Ca}^{2+}]_\text{i}$ changes were represented by $F_i/F_0$. †, addition of CCK-8 ($10^2$ pmol/L).
proteins, and stimulate pancreatic enzyme secretion. The tyrosine kinase inhibitor genistein inhibited the secretory response to CCK\(^{[11]}\). In contrast, tyrosine kinases may regulate not only cellular growth, but also the activity of some ion channels\(^{[12]}\). Recent studies demonstrated that tyrosine kinases inhibitors decreased the cardiac contractile force and affected the ionic currents in cardiomyocytes\(^{[13]}\). In our experiments, pretreatment with proglumide or genistein inhibited the \([\text{Ca}^{2+}]\) elevation induced by CCK-8, suggesting that CCK-8 could activate receptor-operated \(\text{Ca}^{2+}\) channel and increase \([\text{Ca}^{2+}]\), which might be mediated by tyrosine kinase. However, whether the effect of CCK-8 on intracellular \(\text{Ca}^{2+}\) release is mediated by the increase in IP\(_3\), induced by CCK-8 in cardiomyocytes is to be further studied.

In conclusion, CCK-8 can increase \([\text{Ca}^{2+}]\), via activating the receptor-operated \(\text{Ca}^{2+}\) channel, eliciting the influx of \(\text{Ca}^{2+}\), and inducing intracellular \(\text{Ca}^{2+}\) release in isolated guinea pig cardiomyocytes. Such an effect may be mediated by tyrosine kinase.

REFERENCES


