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Dual Effect of H_2O_2 on the Regulation of Cholecystokinin-induced Amylase Release in Rat Pancreatic Acinar Cells

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H_2O_2, a member of reactive oxygen species (ROS), is known to be involved in the mediation of physiological functions in a variety of cell types. However, little has been known about the physiological role of H_2O_2 in exocrine cells. Therefore, in the present study, the effect of H_2O_2 on cholecystokinin (CCK)-evoked Ca^{2+} mobilization and amylase release was investigated in rat pancreatic acinar cells. Stimulation of the acinar cells with sulfated octapeptide form of CCK (CCK-8S) induced biphasic increase in amylase release. Addition of 30 μM H_2O_2 enhanced amylase release caused by 10 pM CCK-8S, but inhibited the amylase release induced by CCK-8S at concentrations higher than 100 pM. An ROS scavenger, 10 μM Mn(III)tetrakis(4-benzoic acid)porphyrin chloride, increased amylase release caused by CCK-8S at concentrations higher than 100 pM, although lower concentrations of CCK-8S-induced amylase release was not affected. To examine whether the effect of H_2O_2 on CCK-8S-induced amylase release was exerted via modulation of intracellular Ca^{2+} signaling, we measured the changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in fura-2 loaded acinar cells. Although 30 μM H_2O_2 did not induce any increase in $[Ca^{2+}]_i$ by itself, it increased the frequency and amplitude of $[Ca^{2+}]_i$ oscillations caused by 10 pM CCK-8S. However, 30 μM H_2O_2 had little effect on 1 nM CCK-8S-induced increase in $[Ca^{2+}]_i$. ROS scavenger, 1 mM N-acetylcysteine, did not affect $[Ca^{2+}]_i$ changes induced by 10 pM or 1 nM CCK-8S. Therefore, it was concluded that 30 μM H_2O_2 enhanced low concentration of CCK-8S-induced amylase release probably by increasing $[Ca^{2+}]_i$ oscillations while it inhibited high concentration of CCK-8S-induced amylase release.

Keywords: H_2O_2, cholecystokinin, amylase release, pancreatic acinar cells

Introduction

Reactive oxygen species (ROS), such as superoxide anion, H_2O_2 and the hydroxyl radical, have been considered as cytotoxic by-products of cellular metabolism. However, recent studies have provided evidences that H_2O_2 serves as a signaling molecule modulating various physiological functions (Dröge, 2002). Cells possess mechanisms that can rapidly synthesize and destroy H_2O_2 in response to receptor stimulations. For example, stimulation of membrane receptors of various growth factors, such as transforming growth factor-β1, platelet-derived growth factor and epithermal growth factor, triggers the rapid and transient production of H_2O_2 (Ohba *et al.*, 1994; Sundaresan *et al.*, 1995; Bae *et al.*, 1997; Bae *et al.*, 2000). H_2O_2 generated in response to the receptor stimulation has been shown to play an important role in regulating various normal cell functions, such as cell proliferation, platelet aggregation and vasodilation (Sobey *et al.*, 1997; Pignatelli *et al.*, 1998; Varela *et al.*, 2004). In addition to this, exogenous addition of H_2O_2 at low concentrations affects the functions of various proteins involved in signal transduction and ion channels (Tournier *et al.*, 1997; Akaishi *et al.*, 2004; Varela *et al.*, 2004).

The exocrine pancreas plays an essential role in the synthesis and secretion of major enzymes for the digestion,

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Control of pancreatic secretion is regulated primarily by cholecystokinin (CCK). The signal transduction pathways linked to CCK receptor activation have been extensively studied in pancreatic acinar cells (Williams, 2001). Stimulation of the CCK receptors, which are coupled to Gq/11-proteins, increases the production of inositol 1,4,5-trisphosphate (IP_3) through the activation of phospholipase C. IP_3 increases intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) both by mobilization of Ca^{2+} from intracellular Ca^{2+} stores and by stimulation of Ca^{2+} entry across the plasma membrane. Recently, the existence of other Ca^{2+} mobilizing second messengers, such as cyclic ADP ribose and nicotinic acid adenine dinucleotide phosphate (NAADP), was also reported to be involved in the CCK-induced Ca^{2+} mobilization (Cancela *et al.*, 2000). An increase in $[Ca^{2+}]_i$ triggers exocytosis of secretory granules containing digestive enzymes (Williams, 2001).

Recently, a great deal of attention has focused on the sensitivity of the mechanisms responsible for Ca^{2+} mobilization to changes in the redox state. H_2O_2 has been shown to enhance the mobilization of Ca^{2+} in many cell types by modifying Ca^{2+} channels, such as L-type Ca^{2+} channels (Akaishi *et al.*, 2004), long transient receptor potential channel 2 (LTRPC2, Wehage *et al.*, 2002), ryanodine-dependent Ca^{2+} channels (Favero *et al.*, 1995) and IP_3-dependent Ca^{2+} channels (Hu *et al.*, 2000). Ca^{2+} pumps such as sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA, Redondo *et al.*, 2004) and plasma membrane Ca^{2+} ATPase (PMCA, Redondo *et al.*, 2004) or enzymes involved in Ca^{2+} signaling pathways, such as phospholipase Cγ1 (PLCγ1, Wang *et al.*, 2001) and phospholipase D (PLD, Servitja *et al.*, 2000).

Given the widespread involvement of H_2O_2 in modulating Ca^{2+} signaling cascade, it is tempting to speculate that amylase release from pancreatic acinar cells, which is regulated mainly by Ca^{2+} signaling, may be modified by H_2O_2.

Therefore, in the present study, we investigated the roles of H_2O_2 in Ca^{2+} signaling and amylase release in rat pancreatic acinar cells. Our results indicate that low, physiologically relevant concentration of H_2O_2 (30 μM) enhances low concentration of CCK-induced $[Ca^{2+}]_i$ oscillations and amylase release, while it inhibits high concentration of CCK-induced amylase release.

Materials and Methods

Preparation of Rat Pancreatic Acinar Cells

Male Sprague-Dawley rats (150-250 g) were anesthetized with diethyl ether and killed by decapitation. The pancreata were immediately removed and trimmed of fat on ice. The acini were prepared using 50 U/ml collagenase by a slight modification of the methods described previously (Matozaki *et al.*, 1990; Toescu *et al.*, 1993). The isolated pancreatic acinar cells were suspended in a HEPES-buffered physiological solution containing (in mM): NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, HEPES free acid 10, D-glucose 11.1, bovine serum albumin (0.1 mg/ml), soybean trypsin inhibitor (0.15 mg/ml) and pyruvic acid (1 mg/ml) (adjusted to pH 7.4, gassed with 100% O_2).

Amylase Assay

Isolated pancreatic acini were centrifuged and then resuspended in fresh HEPES-buffered physiological solution containing 1.5 mM $CaCl_2$. One ml aliquots of acinar suspension distributed into 10 ml polycarbonate Erlenmeyer flasks were incubated with secretagogues at 37°C for 30 min under constant shaking at 60 times/min, and the incubation was then terminated with centrifugation at 500 g for 3 min at 4°C. The supernatant was reserved on ice for a subsequent amylase assay, which was carried out by a modified method of Bernfeld using starch as a substrate (Bernfeld, 1955). The amylase release was expressed as the percentage of the total content of amylase in each sample.

Fura-2 Loading and $[Ca^{2+}]_i$ Measurements

The cells were loaded with fura-2 by incubation with 2 μM acetoxymethyl ester of fura-2 (fura-2/AM) in a HEPES buffered solution equilibrated with 100% O_2 for 40 min at room temperature. They were washed twice and resuspended in a HEPES buffered solution. The cells were allowed to attach to a coverslip that formed the base of a cell chamber mounted on the stage of an inverted microscope and were superfused with the HEPES buffered solution at a flow rate of 2 ml/min. $[Ca^{2+}]_i$ was measured by spectrofluorometry (Photon Technology International, Brunswick, NJ) with excitations at 340 and 380 nm and emission was measured at 510 nm. The values of $[Ca^{2+}]_i$ were calculated from the ratio of fluorescence intensities ($F_{340/380}$) according to Grynkiewicz *et al.* (1985).

Materials

H_2O_2, N-acetylcysteine (NAC), Collagenase (type IV), soybean trypsin inhibitor, HEPES, bovine serum albumin, MEM amino acids, glutamine and sulfated octapeptide form of CCK (CCK-8S) were purchased from Sigma (St. Louis, MO). Mn(Ⅲ)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP) was from Calbiochem (San Diego, CA). Fura-2/AM was obtained from Molecular Probes (Eugene, OR).

Results

Effect of H_2O_2 on CCK-induced Amylase Release

CCK has been known to produce a typical biphasic dose-response curve for amylase release (Stark *et al.*, 1989). In this study, we also observed that the amylase release increased as CCK-8S concentration increased, reaching a maximum at 100 pM, and then decreased (Fig. 1A). To examine the effect of H_2O_2 on amylase release, we treated

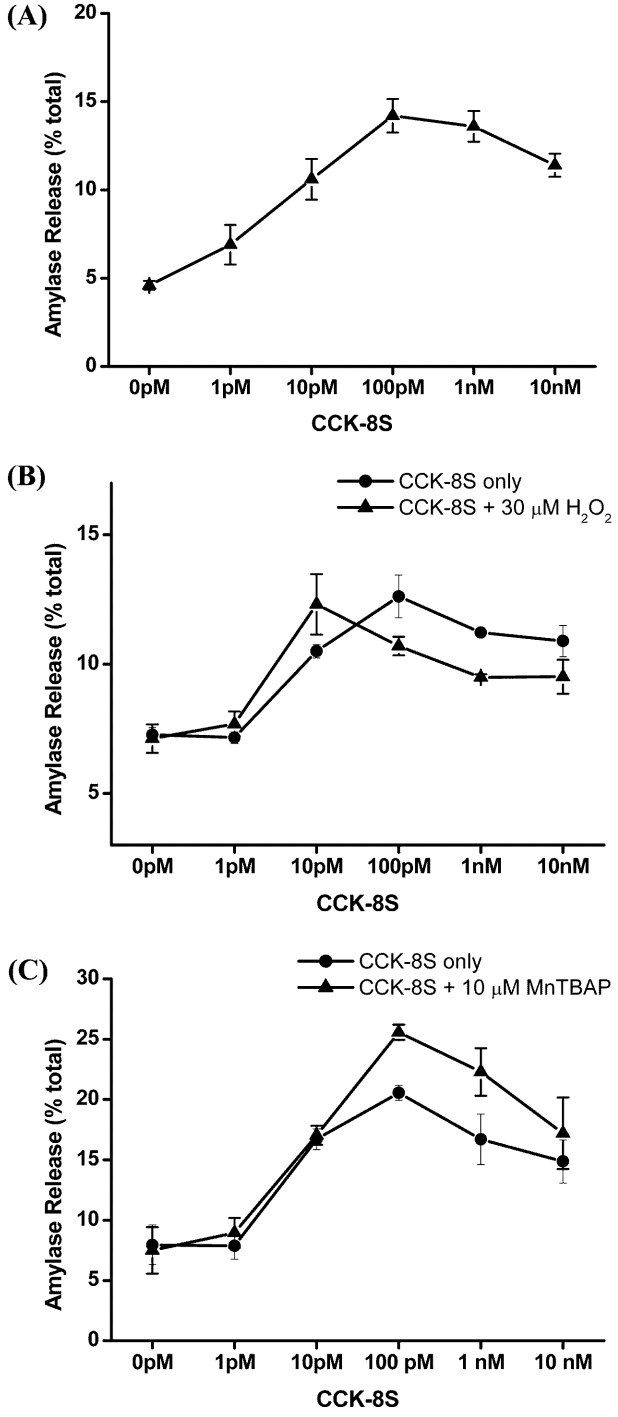

Fig. 1. Dual Effect of H_2O_2 on CCK-induced amylase release in rat pancreatic acinar cells. Cells were exposed to various concentrations of CCK-8S with/without 30 μM H_2O_2 or 10 μM MnTBAP at 37°C for 30 min and the amylase assay was carried out as described in *Materials and Methods*. The amylase release was expressed as the percentage of the total content of amylase in each sample. A. CCK-8S induced a biphasic increase in amylase release. B. Addition of 30 μM H_2O_2 increased 10 pM CCK-8S-induced amylase release, but decreased the amylase release caused by CCK-8S at concentrations higher than 100 pM. C. ROS scavenger 10 μM MnTBAP increased the amylase release caused by CCK-8S at concentrations higher than 100 pM. Values are means±S.E.M. from 6-9 separate experiments.

cells with 30 μM H_2O_2 in combination with each concentration of CCK-8S. As shown in Fig. 1B, addition of 30 μM H_2O_2 increased 10 pM CCK-8S-induced amylase release, but decreased the amylase release caused by CCK-8S at concentrations higher than 100 pM.

Effect of ROS Scavenger on CCK-induced Amylase Release

Because stimulation of the pancreatic acinar cells with CCK has been known to produce ROS (Granados *et al.*, 2004), we examined whether scavenging ROS affected the CCK-induced amylase release. Since some scavengers, such as NAC and glutathione, interfered the measurement of optical density (data not shown), we used MnTBAP to measure the amylase release. As shown in Fig. 1C, 10 μM MnTBAP increased amylase release induced by 100 pM or higher concentrations of CCK-8S, suggesting that ROS produced by CCK-8S stimulation may be involved in the inhibition of high concentrations of CCK-induced amylase release in rat pancreatic acinar cells. The amylase release induced by CCK-8S at concentrations lower than 100 pM was not affect by 10 μM MnTBAP.

Effect of H_2O_2 on $[Ca^{2+}]_i$ Increase in Rat Pancreatic Acinar Cells

The effect of H_2O_2 on Ca^{2+} mobilization was examined in fura-2 loaded rat pancreatic acinar cells. As shown in Fig. 2A and B, exposure of the cells to H_2O_2 at concentrations lower than 100 μM failed to increase $[Ca^{2+}]_i$ at least for 10 min. In contrast, 100 μM H_2O_2 was shown to increase $[Ca^{2+}]_i$ by 42±11.4 nM and 1 mM H_2O_2 induced a rapid increase in $[Ca^{2+}]_i$ by 235±32.6 nM. However, the Ca^{2+} response to 1 mM H_2O_2 was irreversible, suggesting that this concentration of H_2O_2 caused cell damage. H_2O_2, at concentrations lower than 1 mM, did not induce significant increase in amylase release (data not shown).

Effect of H_2O_2 on CCK-induced Ca^{2+} Mobilization

Since Ca^{2+} signaling plays a critical role in the regulation of amylase release in rat pancreatic acinar cells, we checked whether 30 μM H_2O_2 affected CCK-induced Ca^{2+} mobilization. As shown in Fig. 3A, 10 pM CCK-8S induced $[Ca^{2+}]_i$ oscillations and an addition of 30 μM H_2O_2 enhanced the 10 pM CCK-8S-induced $[Ca^{2+}]_i$ oscillations. On the contrary, 30 μM H_2O_2 did not affect Ca^{2+} mobilization caused by 100 pM or higher concentrations of CCK-8S (Fig. 4A and 4B). Scavenging ROS using 1 mM NAC did not affect CCK-8S-induced Ca^{2+} mobilization (Fig. 3B and Fig. 4B).

Discussion

H_2O_2 is a member of ROS, which cause oxidative damage to cellular components such as lipids, nucleic acids, and proteins. Therefore, H_2O_2 has generally been considered to

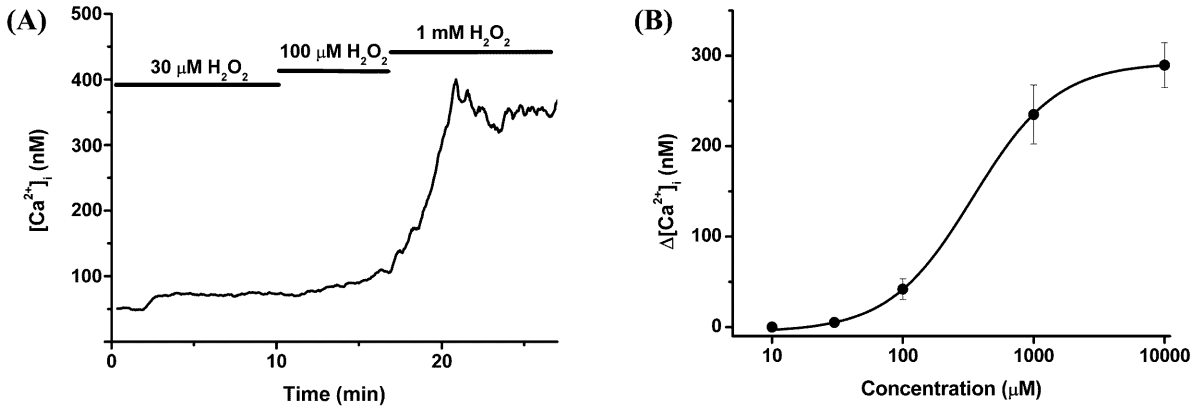

Fig. 2. Dose-dependent Ca^{2+} mobilization by H_2O_2 in rat pancreatic acinar cells. Cells were loaded with fura-2 as described in *Materials and Methods* and changes in $[Ca^{2+}]_i$ were measured using ratiometric fluorescence imaging. (A) Cells were consecutively exposed to 30 μM, 100 μM and 1 mM of H_2O_2. The trace is representative of 18-20 cells in four independent experiments. (B) The size of $[Ca^{2+}]_i$ increases in respond to H_2O_2 is plotted as a function of the added H_2O_2 concentrations (10 μM, 30 μM, 100 μM, 1 mM and 10 mM). Results are depicted as mean ± S.E.M.

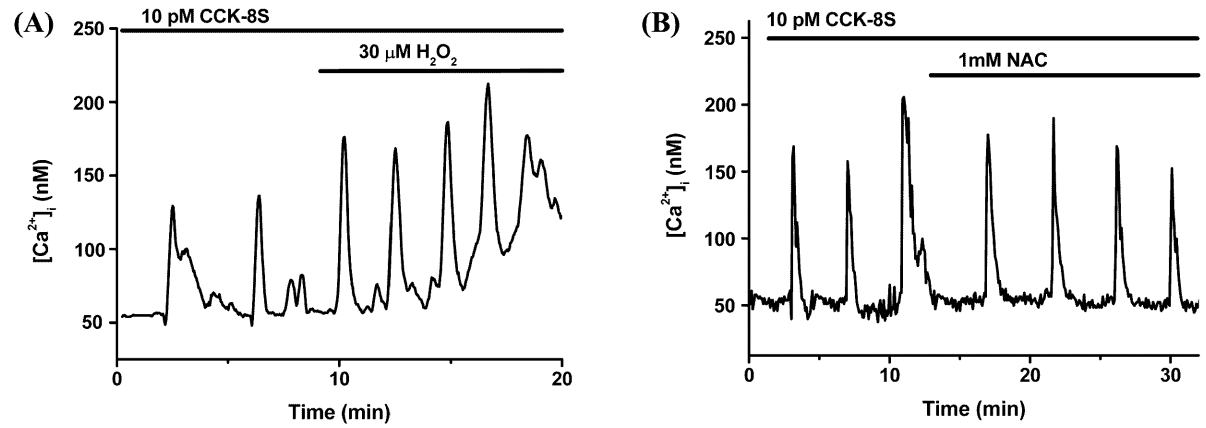

Fig. 3. Effect of H_2O_2 or NAC, an ROS scavenger, on low concentration of CCK-induced $[Ca^{2+}]_i$ oscillations in rat pancreatic acinar cells. Cells were exposed to 10 pM CCK-8S to generate $[Ca^{2+}]_i$ oscillations and then 30 μM H_2O_2 (A) or 1 mM NAC (B) was added. Each trace is representative of 18-20 cells in four independent experiments.

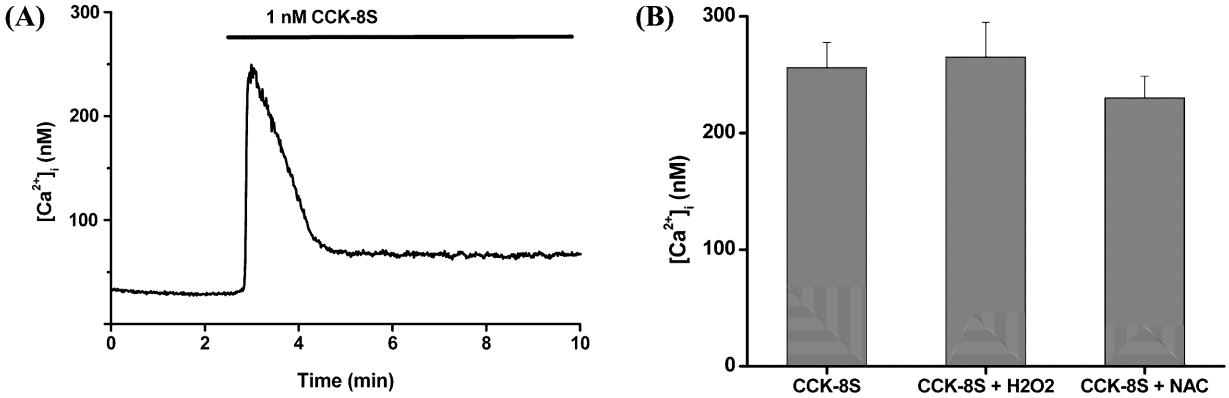

Fig. 4. Effect of H_2O_2 or NAC, an ROS scavenger, on high concentration of CCK-induced $[Ca^{2+}]_i$ increases in rat pancreatic acinar cells. (A) Cells were exposed to 1 nM CCK-8S to induce biphasic $[Ca^{2+}]_i$ increase. The trace is representative of 18-20 cells in four independent experiments. (B) The peak concentrations of intracellular Ca^{2+} achieved in respond to 1 nM CCK-8S in the presence of absence of 30 μM H_2O_2 or 1 mM NAC were measured. Note that $[Ca^{2+}]_i$ increases induced by CCK-8S were not affected by 30 μM H_2O_2 or NAC, an ROS scavenger. Values are means ± S.E.M. from 3-5 separate experiments.

be cytotoxic and hazardous to living organisms. However, H_2O_2 is also known to play a crucial role in the regulation of various physiological functions as an intracellular messenger (Dröge, 2002). The physiologically relevant concentration range of H_2O_2, which causes an acceleration of cellular functions in a variety of cell types, is considered 1-100 μM although it depends on cell type (Gamaley and Klyubin, 1999; Dröge, 2002). Since the main aim of this study was to examine the physiological role of ROS in the regulation of amylase release from pancreatic acinar cells, we used 30 μM H_2O_2.

In the present study, we found that exogenous addition of 30 μM H_2O_2 had a dual effect on amylase release in rat pancreatic acinar cells; 30 μM H_2O_2 enhanced amylase release caused by low concentration of CCK whereas it inhibited high concentration of CCK-induced amylase release. An article published recently by Granados and colleagues (2006) also showed that H_2O_2 exerted an inhibitory effect on CCK-induced amylase secretion. However, our findings are contrary to those reported by Granados *et al*., which demonstrated that H_2O_2 inhibited amylase releases caused by both low and high concentration of CCK. One possible explanation for this discrepancy would be the use of different animal species. Further study might be necessary for elucidating the differences.

Amylase release triggered by CCK receptor activation is biphasic; CCK increased amylase release in a dose-dependent manner but concentrations above 1 nM caused a reduction in amylase release. This is because the rat pancreatic acinar cells possess two different receptor states, high affinity receptors and low affinity receptors (Williams and Blevins, 1993). The activation of the high affinity CCK receptors enhances amylase release, whereas the low affinity CCK receptor causes the inhibition of amylase release.

Activation of the CCK receptors is also known to induce two different patterns of Ca^{2+} responses. The high affinity CCK receptors have been known to elicit $[Ca^{2+}]_i$ oscillations, while the low affinity CCK receptors are responsible for the biphasic increase in $[Ca^{2+}]_i$, consisting of a rapid increase in $[Ca^{2+}]_i$ followed by a slow decrease to a sustained elevated level (Matozaki *et al*., 1990).

Ca^{2+} plays a pivotal role in the regulation of a diverse range of cellular functions, such as muscle contraction, secretion, synaptic plasticity, cell proliferation and cell death (Berridge *et al*., 2000). Cellular Ca^{2+} signals generally encode information in two different modes; frequency-modulated and amplitude-modulated signals (Berridge, 1997). Frequency-modulated Ca^{2+} signaling (i.e. Ca^{2+} oscillations) is generally considered to have the highest fidelity, and many cells use this paradigm in response to low physiological concentrations of agonists (Berridge *et al*., 1998).

Since we also observed that low concentration of CCK-8S induced $[Ca^{2+}]_i$ oscillations and amylase release, we first examined whether H_2O_2-enhanced amylase release was due to the increase in the CCK-induced $[Ca^{2+}]_i$ oscillations. Although 30 μM H_2O_2 did not mobilize Ca^{2+} by itself in our system, it obviously enhanced the CCK-induced $[Ca^{2+}]_i$ oscillations. Pancreatic acinar cells are not unique in this respect. H_2O_2 was also shown to enhance $[Ca^{2+}]_i$ oscillations induced by histamine in human aortic endothelial cells (Hu *et al*., 2002). In this cell type and rat astrocytes, H_2O_2 was shown to induce $[Ca^{2+}]_i$ oscillations by itself. The mechanisms by which H_2O_2 enhances or induces $[Ca^{2+}]_i$ oscillations in various cells are controversial. In endothelial cells, increasing the sensitivity of endoplasmic reticulum (ER) to IP_3 was suggested to be responsible for the H_2O_2-induced $[Ca^{2+}]_i$ oscillations (Hu *et al*., 2000; 2002). In contrast to this, increased IP_3 production by the activation of PLCγ1 was shown to play a critical role in the H_2O_2 induced $[Ca^{2+}]_i$ oscillations in cultured rat astrocytes (Hong *et al*., 2006). Although the mechanism responsible for the enhancement of CCK-induced $[Ca^{2+}]_i$ oscillations by H_2O_2 was not elucidated in this study, both increased IP_3 production and sensitivity of ER to IP_3 could be involved.

Although H_2O_2 increased low concentration of CCK-induced amylase release, it did not increase the amylase release induced by high concentrations of CCK. Instead, it decreased the amylase release. In addition to this, MnTBAP was shown to increase amylase release induced by high concentrations of CCK. Since it has been shown that high concentration of CCK accumulates ROS in rat pancreatic acinar cells (Granados *et al*., 2004), our data suggest that ROS produced by high concentration of CCK may contribute to the inhibition of amylase release. The mechanism for the inhibition of amylase release by H_2O_2 was not clear, but our data indicated that high concentration of CCK-induced Ca^{2+} mobilization was not affected. Therefore, H_2O_2 might act on the downstream signal of $[Ca^{2+}]_i$ increase, leading to the inhibition of exocytosis of the secretory granules. In support of our observation, Rosado and colleagues reported that H_2O_2 induced temporal and spatial modifications in actin filament reorganization, which was responsible for the decrease in the CCK-stimulated pancreatic secretion (Rosado *et al*., 2002). The exact mechanism for the inhibition of high-concentration of CCK-induced amylase release caused by H_2O_2 remains to be elucidated.

Therefore, it was concluded that, although 30 μM H_2O_2 did not induce $[Ca^{2+}]_i$ oscillations by itself, it increased the low concentrations of CCK-induced $[Ca^{2+}]_i$ oscillations and amylase release. However, 30 μM H_2O_2 was shown to inhibit the amylase release caused by high concentrations of CCK and the inhibitory effect did not appear to be mediated by inhibition of Ca^{2+} signaling. A further study will be needed to elucidate the inhibitory mechanisms.

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