

Low affinity cholecystokinin receptor inhibits cholecystokinin- and bombesin-induced oscillations of cytosolic Ca^{2+} concentration

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Abstract We have investigated whether low affinity cholecystokinin (CCK) receptors suppress agonist-induced rises of cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$) in pancreatic acinar cells by using properties of caffeine. A high concentration of caffeine (20 mM) completely blocked inositol 1,4,5-trisphosphate (InsP_3)-induced $[\text{Ca}^{2+}]_c$ rises but spared the InsP_3 -independent long-lasting $[\text{Ca}^{2+}]_c$ oscillations. In the presence of 20 mM caffeine, only high concentrations of CCK, but not bombesin or JMV-180, suppressed the caffeine-resistant CCK or bombesin-induced $[\text{Ca}^{2+}]_c$ oscillations, indicating that low affinity CCK receptors inhibit agonist-induced $[\text{Ca}^{2+}]_c$ oscillations. It could be one of the underlying mechanisms by which low affinity CCK receptors suppress secretion in pancreatic acinar cells.

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Key words: Cholecystokinin; Bombesin; Caffeine; Pancreatic acinar cell; Cytosolic free Ca^{2+} concentration; Low affinity cholecystokinin receptor

1. Introduction

In pancreatic acinar cells, many secretagogues give rise to cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$) to trigger opening of Ca^{2+} -activated Cl^- channels and secretion of zymogen granules [1–5]. Cholecystokinin (CCK), bombesin, and acetylcholine (ACh) are well known secretagogues in pancreatic acinar cells and also generate Ca^{2+} release from intracellular Ca^{2+} stores by activating many but different intracellular second messengers [4–7]. So far it is likely that ACh exclusively produces inositol 1,4,5-trisphosphate (InsP_3) as a second messenger to activate Ca^{2+} signals but CCK and bombesin recruit more second messengers such as cyclic ADP-ribose and nicotinic acid adenine dinucleotide phosphate [4,5,8,9]. Among them, CCK is reported to act on two kinds of different receptor states, known as low and high affinity CCK receptors, depending on its concentrations [2,10–14]. High affinity CCK receptors, which can be activated by CCK at low concentrations or JMV-180 (a specific agonist), appear to elicit various $[\text{Ca}^{2+}]_c$ oscillations through complex interplay between several kinds of second messengers without a significant elevation of InsP_3 concentration [15–18]. However, it has been reported that, when cells are minimally stimulated with a very low concentration of CCK (2–10 pM), short-lasting (a few seconds

or shorter) $[\text{Ca}^{2+}]_c$ oscillations can be monitored by measuring Ca^{2+} -sensitive currents under the whole-cell patch-clamp configuration and the Ca^{2+} -sensitive currents depend on functional InsP_3 receptors [4,9,19]. On the other hand, the low affinity CCK receptors appear to have dual opposite actions on the pancreatic secretions; in addition to raising $[\text{Ca}^{2+}]_c$ through InsP_3 productions [15–18], they also inhibit amylase secretions through unclear or multiple mechanisms in pancreatic acinar cells [2,14,18,20]. Since a $[\text{Ca}^{2+}]_c$ rise is the most important single factor for determining exocytosis, low affinity CCK receptors may suppress amylase secretion by directly inhibiting agonist-induced Ca^{2+} signals. But this possibility has not been tested, since the inhibitory signal of the low affinity CCK receptors cannot be selectively activated unless activating the high affinity CCK receptors as well as the low affinity CCK receptors linked with InsP_3 , due to the lack of a specific agonist which selectively activates inhibitory signals of the low affinity CCK receptors. Therefore, if cells are exposed to high concentrations of CCK, the $[\text{Ca}^{2+}]_c$ rises high through the combined mechanisms of low and high affinity CCK receptors activated. In this condition, it is hardly difficult to selectively observe the inhibitory action of low affinity CCK receptors on the $[\text{Ca}^{2+}]_c$ changes. Additionally, it is not known whether low affinity CCK receptors modulate the Ca^{2+} signal that is evoked by another major secretagogue, bombesin.

Generally, it is well known that many Ca^{2+} mobilizing secretagogues such as ACh, CCK, and bombesin cause Ca^{2+} release from the endoplasmic reticulum through the two kinds of intracellular Ca^{2+} channels, InsP_3 and ryanodine receptors [3–5]. Since caffeine potently inhibits the InsP_3 pathway in the pancreatic acinar cells [9,18,21], it has long been used as a blocker for Ca^{2+} release through InsP_3 receptors [4]. We took this advantage to selectively activate the inhibitory signal of the low affinity CCK receptors without activating InsP_3 pathways by applying high concentrations of CCK in the presence of a high concentration of caffeine (20 mM). Since CCK and bombesin in the presence of 20 mM caffeine could, nevertheless, generate the caffeine-resistant long-lasting $[\text{Ca}^{2+}]_c$ oscillations unlike ACh, we could demonstrate that activation of low affinity CCK receptors suppresses the CCK or bombesin-induced long-lasting $[\text{Ca}^{2+}]_c$ oscillations.

2. Materials and methods

2.1. Cell preparation

Single isolated mouse (Balb/c) pancreatic acinar cells were prepared using collagenase digestion as described previously [22–23].

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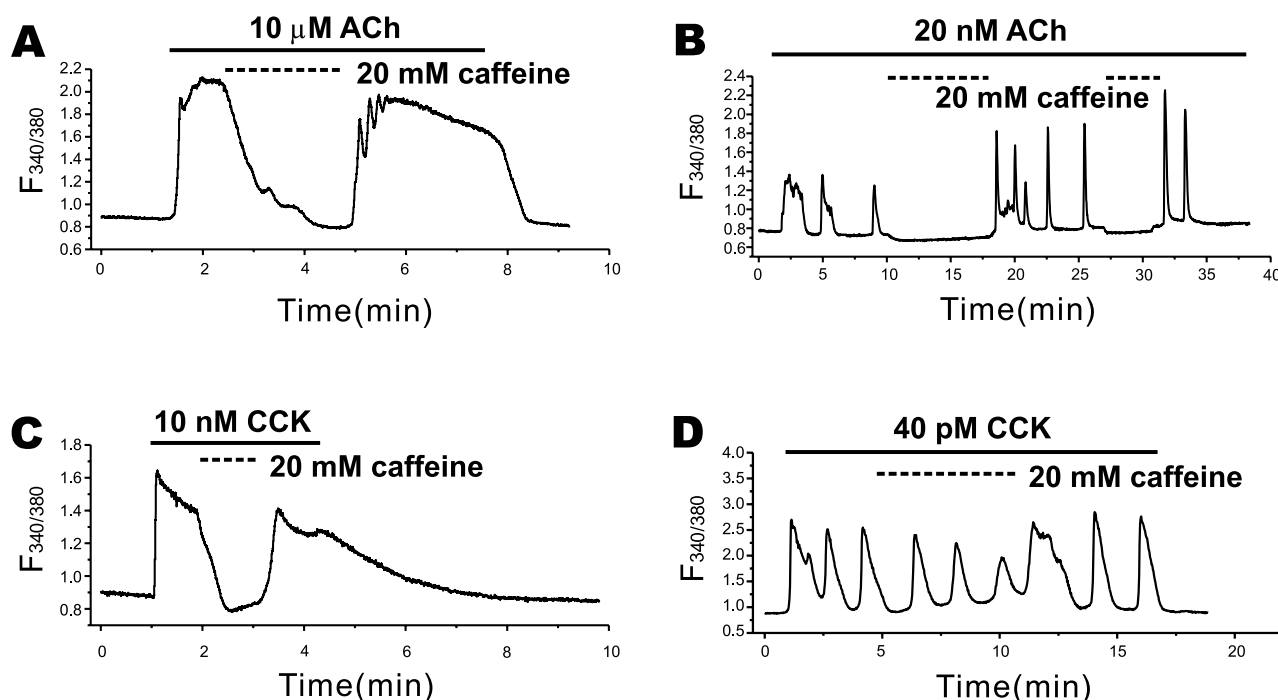


Fig. 1. Effects of caffeine on the ACh- or CCK-induced Ca^{2+} signals. A: Application of 20 mM caffeine completely blocked 10 μ M ACh-induced sustained $[\text{Ca}^{2+}]_i$ rise ($n=10$). B: Caffeine (20 mM) blocked 20 nM ACh-induced $[\text{Ca}^{2+}]_i$ oscillations ($n=7$). C: Caffeine (20 mM) blocked 10 nM CCK-generated sustained $[\text{Ca}^{2+}]_i$ rises ($n=6$). D: Caffeine (20 mM) failed to block 40 pM CCK-induced $[\text{Ca}^{2+}]_i$ oscillations ($n=12$).

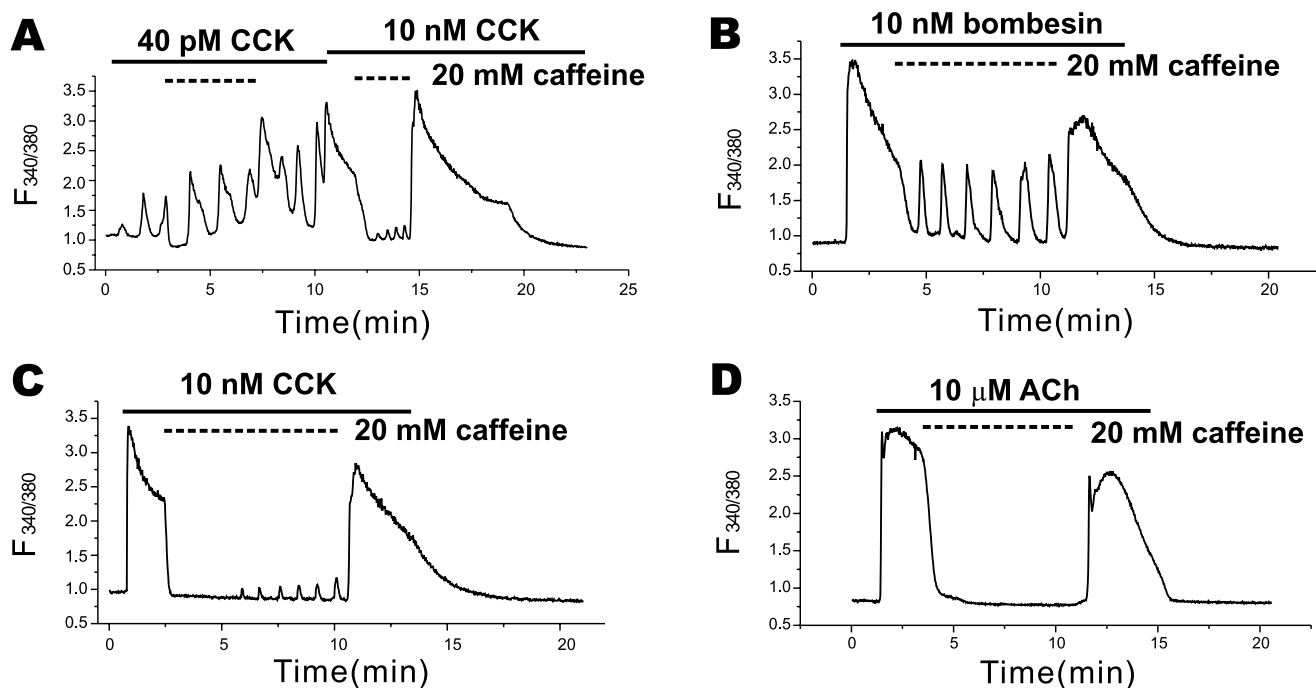


Fig. 2. Different Ca^{2+} signals between CCK, ACh and bombesin. A: Caffeine (20 mM) did not block the 40 pM CCK-induced $[\text{Ca}^{2+}]_i$ oscillation but blocked the 10 nM CCK-induced $[\text{Ca}^{2+}]_i$ rise. Similar responses from 14 out of 17 cells. B: In a 10 nM bombesin-treated cell, caffeine (20 mM) blocked only the sustained $[\text{Ca}^{2+}]_i$ rise but spared the $[\text{Ca}^{2+}]_i$ oscillations. Similar responses from 14 out of 16 cells. C: The $[\text{Ca}^{2+}]_i$ oscillations were slowly revealed in the presence of 20 mM caffeine. Similar responses from 8 out of 10 cells. D: ACh-induced $[\text{Ca}^{2+}]_i$ rises were completely blocked by 20 mM caffeine ($n=9$).

2.2. Solutions

The extracellular bathing solution contained (in mM): NaCl 140, KCl 4.7, MgCl₂ 1.13, CaCl₂ 1, glucose 10 and HEPES 10, and pH 7.4 adjusted by NaOH. The cells, placed on a glass cover slip attached to an open perfusion chamber, were continuously perfused from a gravity-fed system. All experiments were performed at room temperature (20–24°C). Fura-2-AM and pluronic F-127 were purchased from Molecular Probes and all the other chemicals are from Sigma Co.

2.3. Measuring $[Ca^{2+}]_c$

The isolated pancreatic acinar cells were incubated with 2–5 μ M fura-2-AM at room temperature (20–24°C) for 35 min with 100 mg/l pluronic F-127. After that, the cells were washed with normal physiological salt solution after centrifugation (1000 rpm, 1 min) twice, and then kept in a 4°C refrigerator until use. All cells were used within 4 h after isolation. Single cell fluorescence intensity was measured using a Olympus IX70 inverted microscope (40 $\times$ objective or 60 $\times$ water immersion objective), attached with a charge coupled device image intensifier camera (Quantix) and Metafluor software (Universal Imaging). We used 340/380 dual excitations with a 400 nm dichroic mirror and emitted light was collected with a long-pass filter of 450 nm. Detailed procedures were described previously [22–23].

3. Results and discussion

3.1. Effects of caffeine on ACh and CCK-induced cytosolic Ca^{2+} signals

ACh is well known to generate InsP₃ through activation of G-proteins in pancreatic acinar cells [1–5]. Since the $[Ca^{2+}]_c$ increase in pancreatic acinar cells by ACh is completely blocked by application of caffeine (a blocker for InsP₃ production, see [21]) as well as by the inclusion of heparin (membrane-impermeable inhibitor for InsP₃ receptor) in the pipette solution [9], it is likely that ACh-induced Ca^{2+} signals in this cell are exclusively triggered and dominated by InsP₃ receptors [4,9]. Although caffeine is initially known to activate ryanodine receptors in many cells [24,25], it turns out to be a potent blocker for the InsP₃ pathway in pancreatic acinar cells by inhibiting the InsP₃ receptor itself [26] and InsP₃ productions [9,21,27]. Since caffeine is membrane-permeable and completely blocks the InsP₃-mediated $[Ca^{2+}]_c$ rise [9], it looks to be a good tool in investigating Ca^{2+} signals.

At first, to confirm this, we stimulated cells with ACh and applied a high concentration of caffeine (20 mM). As shown in Fig. 1, high and low concentrations of ACh generated two kinds of Ca^{2+} signals; the sustained huge $[Ca^{2+}]_c$ rise with strong stimulations or the long-lasting $[Ca^{2+}]_c$ oscillations with submaximal stimulations. Generally the short-lasting $[Ca^{2+}]_c$ oscillations (generated by minimal stimulations) appear to be confined to the apical granular areas of the cell and linger a few seconds or shorter, thus the whole-cell patch-clamp technique is employed [3,4,9,19]. However, in the microfluorimetric systems, these short-lasting $[Ca^{2+}]_c$ oscillations are hardly detected but the long-lasting $[Ca^{2+}]_c$ oscillations were usually observed in our experimental conditions and also others [18,28,29,30]. Application of 20 mM caffeine completely blocked both the sustained $[Ca^{2+}]_c$ rises and the $[Ca^{2+}]_c$ oscillations evoked by high and low concentrations of ACh (Fig. 1A,B). This result is completely compatible with the above reports implying that ACh is exclusively involved in the InsP₃ pathway and caffeine is an effective blocker for InsP₃ pathways in pancreatic acinar cells. However, when cells were stimulated with CCK, the response was different. In cells stimulated with 10 nM CCK, 20 mM caffeine completely blocked the $[Ca^{2+}]_c$ rise (Fig. 1C), but it paradoxically did not block the long-lasting $[Ca^{2+}]_c$ oscillations evoked

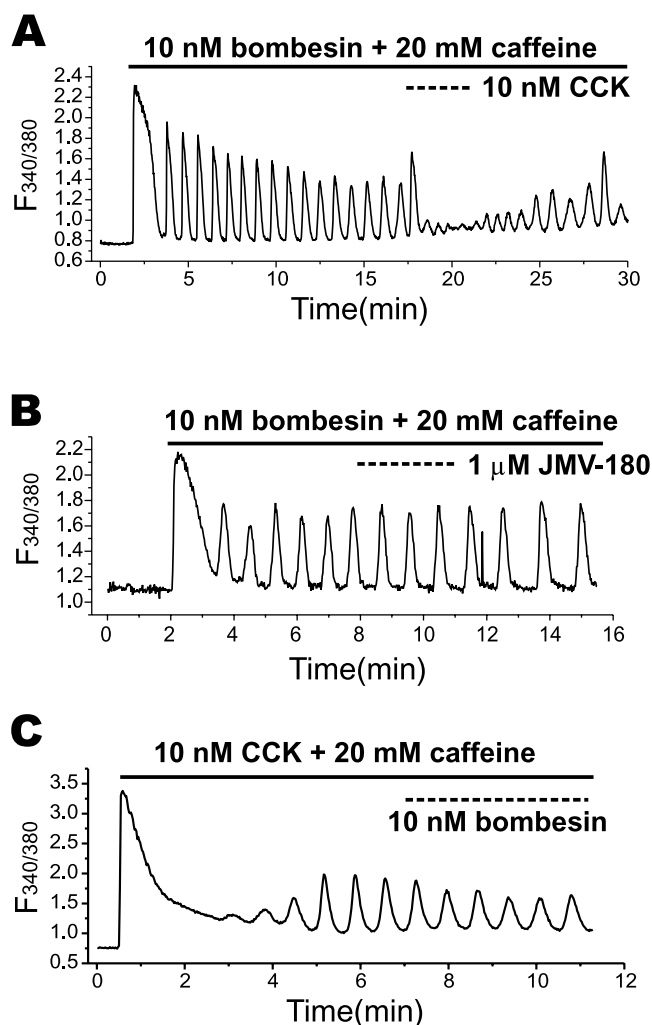


Fig. 3. Interactions between CCK and bombesin. In the presence of 20 mM caffeine, the InsP₃-independent $[Ca^{2+}]_c$ oscillations were induced by applications of supramaximal concentrations (10 nM) of CCK and bombesin. A: CCK suppressed bombesin-induced $[Ca^{2+}]_c$ oscillations ($n=8$). B: JMV-180 (1 μ M) did not affect bombesin-induced $[Ca^{2+}]_c$ oscillations ($n=6$). C: Bombesin did not affect CCK-induced $[Ca^{2+}]_c$ oscillations ($n=6$).

with a low concentration of CCK (40 pM, Fig. 1D). It is interesting that caffeine showed a more dramatic blockade when cells were exposed to 10 nM CCK rather than 40 pM CCK.

To confirm whether this paradoxical blockade is not ascribed to the variable sensitivity of the cells to caffeine and CCK, we serially stimulated the same cells with at first 40 pM and later 10 nM CCK and applied 20 mM caffeine during each stimulation. As shown in Fig. 2A, caffeine did not block the 40 pM CCK-induced $[Ca^{2+}]_c$ rise (oscillatory) but did block the 10 nM CCK-induced $[Ca^{2+}]_c$ rise.

3.2. Different effects of caffeine on CCK- and bombesin-induced Ca^{2+} signals

To see whether this paradoxical blocking effect is occurring in the Ca^{2+} signals evoked by other agonists, we stimulated cells with high concentrations of bombesin and ACh, respectively. As shown in Fig. 2B, after elevating $[Ca^{2+}]_c$ with a high concentration of bombesin, we applied 20 mM caffeine during the stimulation. Interestingly caffeine, unlike CCK, only

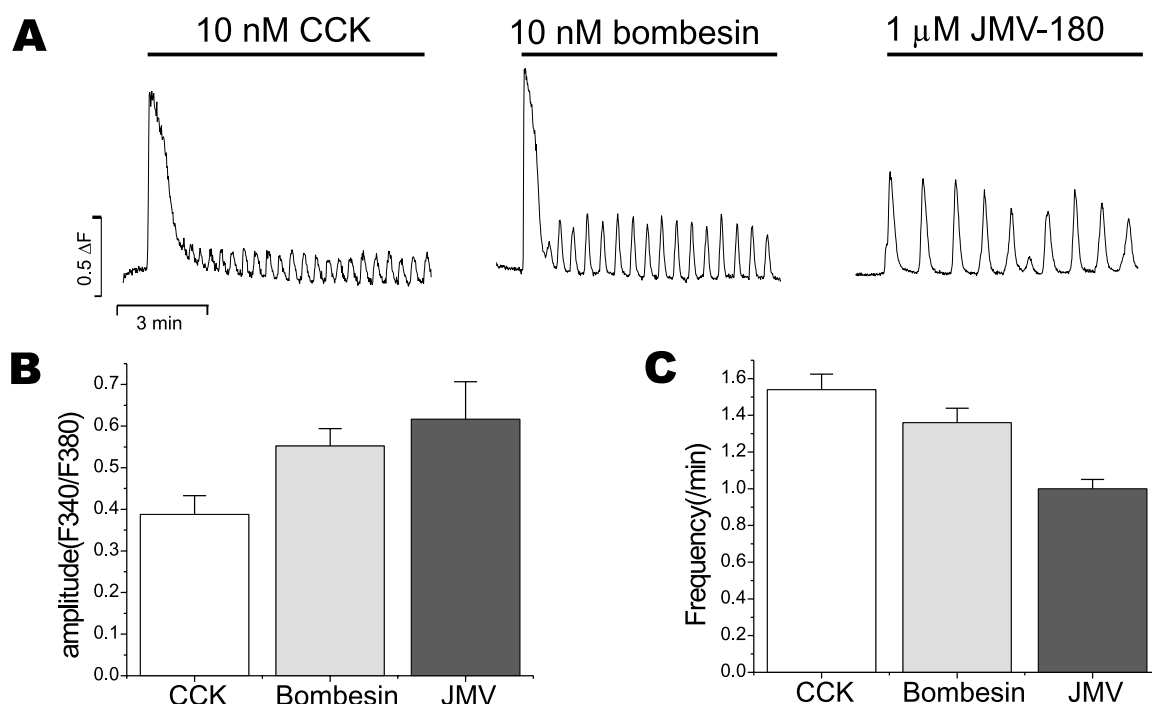


Fig. 4. Patterns of CCK, bombesin, and JMV-180-induced $[Ca^{2+}]_c$ oscillations in the presence of caffeine. Cells were incubated with 20 mM caffeine for 10 min. A: Typical patterns of $[Ca^{2+}]_c$ oscillations induced by ACh (10 μ M), bombesin (10 μ M) and JMV-180 (1 μ M). B: Comparison of frequencies and amplitudes between the $[Ca^{2+}]_c$ oscillations evoked by CCK ($n=19$), bombesin ($n=27$), and JMV-180 ($n=6$). Statistically significant differences with Student's *t*-test ($P<0.05$) between each column.

blocked the sustained $[Ca^{2+}]_c$ rise but spared the $[Ca^{2+}]_c$ oscillations which are usually occurring at low concentrations of bombesin. Since 20 mM caffeine also did not block the $[Ca^{2+}]_c$ oscillations evoked by a low dose of bombesin (data not shown, $n=6$) like CCK (Fig. 1D), we conclude that CCK and bombesin at low concentrations can generate $InsP_3$ -independent long-lasting $[Ca^{2+}]_c$ oscillations. However, they at high concentrations appear to generate the $InsP_3$ -dependent sustained $[Ca^{2+}]_c$ elevations since caffeine selectively blocked the $[Ca^{2+}]_c$ rises evoked by high concentrations of CCK and bombesin. In the case of CCK, high concentrations of CCK appear to have another inhibitory action on the $[Ca^{2+}]_c$ oscillations which are usually occurring at submaximal stimulations with low agonist concentrations. This effect does not seem to be related to the potency of caffeine in blocking $InsP_3$ pathways, since the $[Ca^{2+}]_c$ rises evoked by ACh is completely blocked by 20 mM caffeine whatever the concentrations are (Fig. 1A,B, Fig. 2D).

Therefore, the more stronger inhibitory effect of caffeine on the $[Ca^{2+}]_c$ rise evoked by high concentrations of CCK would be interpreted by the presence of another inhibitory mechanism which would be activated at high concentrations of CCK. This could be more clearly demonstrated at Fig. 2C. After generating the sustained $[Ca^{2+}]_c$ elevation with 10 nM CCK, long application of 20 mM caffeine showed an initial complete block of the sustained $[Ca^{2+}]_c$ rises as well as the $[Ca^{2+}]_c$ oscillations, unlike bombesin (Fig. 2B), but later the $[Ca^{2+}]_c$ oscillations were slowly revealed. Among 10 cells we tested, eight cells showed the slow recovery of the $[Ca^{2+}]_c$ oscillations and only two cells showed no $[Ca^{2+}]_c$ oscillations. It suggests that the inhibitory signal of the low affinity CCK receptors is not strong and partially released after its activation. Since it was not seen in the cases of bombesin (Fig. 2B)

or ACh (Fig. 2D), it is likely that this phenomenon is unique to CCK.

3.3. Interaction between CCK and bombesin

From the above data, it is clear that CCK works differently from ACh and bombesin. ACh and bombesin could increase $[Ca^{2+}]_c$ with oscillatory or sustained patterns of the $[Ca^{2+}]_c$ rise depending on their concentrations and did not have any inhibitory signals. However, CCK appeared to have both stimulatory and inhibitory signals and the inhibitory signal seemed to be linked with a low affinity CCK receptors.

Next we tested whether the activation of low affinity CCK receptors inhibits the Ca^{2+} signals generated by another important secretagogue, bombesin. As shown in Fig. 3A, we could generate $[Ca^{2+}]_c$ oscillations with a supramaximal concentration of bombesin (10 nM) in the presence of 20 mM caffeine. After that, we suddenly applied a supramaximal concentration of CCK (10 nM) to activate low affinity CCK receptors. Interestingly application of 10 nM CCK inhibited the bombesin-induced $[Ca^{2+}]_c$ oscillations, suggesting that low affinity CCK receptors suppress bombesin-induced Ca^{2+} signals too. It is compatible with the recent report that CCK can inhibit bombesin-induced amylase secretion [31], although they did not envisage the involvement of Ca^{2+} signals.

To rule out the possibility that high affinity CCK receptors may be involved in this inhibitory signal, we used JMV-180, a high affinity CCK receptor agonist. As shown in Fig. 3B, 1 μ M JMV-180 had no effect on the 10 nM bombesin-induced $[Ca^{2+}]_c$ oscillations. In addition, in the presence of 20 mM caffeine, application of 10 nM bombesin (supramaximal) also had no effect on the 10 nM CCK-induced $[Ca^{2+}]_c$ oscillations at all (Fig. 3C), suggesting that the inhibitory signal is specific to the low affinity CCK receptors. At a separate experiment,

JMV-180 easily generated $[Ca^{2+}]_i$ oscillations and caffeine did not block the JMV-180-induced $[Ca^{2+}]_i$ oscillations (data not shown, $n=6$), suggesting that JMV-180 mimics the $[Ca^{2+}]_i$ oscillations evoked by high affinity CCK receptors. Moreover, 1 μ M bisindolmaleimide, a PKC inhibitor, had no effect on the $[Ca^{2+}]_i$ oscillations which were evoked with supramaximal concentrations of CCK and bombesin in the presence of 20 mM caffeine (data not shown, $n=6$), suggesting that this inhibitory effect is not mediated through the activation of protein kinase C.

3.4. Patterns of $[Ca^{2+}]_i$ oscillations evoked by CCK, bombesin, and JMV-180 in the presence of caffeine

From the above data, it is likely that ACh, bombesin, and CCK have different signals to generate $[Ca^{2+}]_i$ elevations. Thus in order to compare the different patterns of Ca^{2+} signals, we generated $[Ca^{2+}]_i$ oscillations by applying supramaximal concentrations of CCK, bombesin, and JMV-180 after incubation of cells with 20 mM caffeine. In Fig. 4, we depicted the representative traces and analyzed the frequency and amplitude of each $[Ca^{2+}]_i$ oscillation.

Interestingly, JMV-180 generated the $[Ca^{2+}]_i$ oscillations having a high amplitude and low frequency when compared with those of 10 nM CCK in the presence of 20 mM caffeine. It may be related to the condition of 10 nM CCK +20 mM caffeine in which the high affinity CCK receptors and inhibitory signals of low affinity CCK receptors are activated while the $InsP_3$ pathway involved in a low affinity CCK receptors is blocked. Due to the inhibitory signal, the amplitudes of the $[Ca^{2+}]_i$ oscillations evoked by 10 nM CCK would be smaller. Interestingly the amplitude and frequency of $[Ca^{2+}]_i$ oscillations induced by 10 nM bombesin were between those of $[Ca^{2+}]_i$ oscillations generated by 10 nM CCK and 1 μ M JMV-180. This difference may suggest that the signaling pathways of those secretagogues are not identical. However, if looking at Fig. 3C in which supramaximal stimulation with bombesin had no effect on the $[Ca^{2+}]_i$ oscillations evoked by a supramaximal stimulation with CCK in the presence of 20 mM caffeine, it may suggest that bombesin signals would share parts of the CCK signals. However, unfortunately it is still not clear how each signal is composed of with these data. Anyway, from these data, it is clear that activation of low affinity CCK receptors suppress agonist-induced Ca^{2+} signals which are important in amylase secretions in pancreatic acinar cells [5,9]. As for inhibitory mechanisms of low affinity CCK receptors in pancreatic acinar cells, cytoskeleton [31], protein kinase C [28], members of the mitogen-activated protein kinase family, and p125PAK focal adhesion kinase [2,5,14,32] have been reported. But we provide here evidence that low affinity CCK receptors, in addition to producing $InsP_3$ as a stimulatory signal [15–17], appear to have an inhibitory signal to suppress the agonist-induced $[Ca^{2+}]_i$ oscillations through $InsP_3$ -independent and PKC-independent mechanisms. It could be one of the mechanisms by which low affinity CCK receptors inhibit secretion in pancreatic acinar cells.

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