Potentiation of Cholecystokinin-Induced Amylase Release by Peptide VIP in Guinea Pig Pancreatic Acini

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Abstract The mechanism of the potentiating effect of vasoactive intestinal peptide (VIP) on cholecystokinin (CCK-8)-induced amylase release was studied in isolated and perfused pancreatic acini of the guinea pig. VIP (30 pM–10 nM) potentiated CCK-8 (100 pM)-induced amylase release. Unexpectedly, VIP inhibited CCK-8-induced intracellular Ca\(^{2+}\) oscillations. Forskolin (10 \(\mu\)M), an activator of adenylate cyclase, potentiated CCK-8 (100 pM)-induced amylase release with a time course similar to that observed with VIP. Caffeine (20 mM) inhibited both amylase release and Ca\(^{2+}\) oscillations in response to CCK-8, suggesting that inhibition of Ca\(^{2+}\) oscillations does not necessarily lead to a potentiation of amylase release. When intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_c\)) was raised by thapsigargin (10 \(\mu\)M), a selective inhibitor of Ca\(^{2+}\)-ATPase in the endoplasmic reticulum (ER), VIP (10 nM) induced significantly greater amylase release than that induced by VIP alone. When [Ca\(^{2+}\)]\(_c\) was lowered by preincubation with BAPTA-AM (25 \(\mu\)M), a cell-permeant Ca\(^{2+}\) chelator, VIP-induced amylase release was completely abolished. These results suggest that VIP, in spite of its inhibitory action on Ca\(^{2+}\) oscillations, facilitates a Ca\(^{2+}\)-dependent process distal to the increase in [Ca\(^{2+}\)]\(_c\) to potentiate CCK-8-induced amylase release.

Key words: cAMP, fura-2, exocrine, potentiation.

Vasoactive intestinal polypeptide (VIP) has been found in neurons innervating the pancreas [1], and VIP receptors have been identified in pancreatic acinar cells as well as duct cells [2, 3]. In addition, it has been shown that exogenously added VIP increased not only pancreatic juice flow but also pancreatic enzyme secretion [4]. This increase in enzyme secretion appears to be a direct effect on acinar cells but not to an indirect effect through the wash out of the pancreatic duct, since VIP-induced increase in amylase release was observed in isolated acinar cells [5] or acini preparations [6]. Moreover, electrical stimulation of the nerves innervating the pancreas increased amylase release in the presence of a cholinergic receptor.

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antagonist [7]. This line of evidence strongly suggests that VIP plays an important role in controlling pancreatic enzyme secretion as a physiological secretagogue.

VIP has been shown to increase the concentration of intracellular cAMP [8] without increasing cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_c\)) [9, 10], whereas the other known physiological secretagogues for pancreatic enzyme secretion, cholecystokinin (CCK) and acetylcholine (ACh), are reported to increase [Ca\(^{2+}\)]\(_c\) in pancreatic acinar cells of the guinea pig [11] as well as of the rat and the mouse [12–14] without changing cytosolic cAMP concentration [15]. Thus, there seem to be two independent signal transduction pathways both of which lead to the stimulation of pancreatic enzyme secretion. One important question is what is the physiological significance of these two pathways, namely, whether there is a crosstalk between these two pathways to effectively evoke pancreatic enzyme secretion. Several studies have shown that VIP potentiates CCK-induced pancreatic enzyme secretion when both are added together [4–6, 15, 16]. However, the mechanism underlying this potentiation between VIP and CCK remains unclear. One possibility is that VIP, acting through phosphorylation via the cAMP-dependent protein kinase A (PKA) pathway, potentiates CCK-induced enzyme secretion by amplifying the CCK-induced [Ca\(^{2+}\)]\(_c\) dynamics. Indeed, such idea was proposed in an early report that revealed potentiation of CCK-induced enzyme secretion by the cAMP-elevating agent, forskolin [17]. Here, we have studied the mechanism of the synergism between VIP and CCK-8 in isolated and perfused pancreatic acini of the guinea pig.

**MATERIALS AND METHODS**

**Materials.** Male Hartley strain guinea pigs (350–450 g) were obtained from Nihon S.L.C., Shizuoka, Japan; Eagle’s minimum essential medium amino acids (MEM) from Flow Laboratories, Irvine, Scotland; vitamin solution from GIBCO Laboratories, Grand Island, NY, U.S.A.; bovine serum albumin (BSA; fraction V) from Sigma Chemical or Irvine Scientific, Santa Ana, Ca, U.S.A.; trypsin inhibitor (TI) from Sigma Chemical; CCK-8 from Peptide Institute, Osaka, Japan; guinea pig VIP was a gift from Dr. N. Yanihara, School of Pharmaceutical Sciences, University of Shizuoka, Shizuoka, Japan; Cell-Tak from Collaborative Biomedical Products, Bedford, MA, U.S.A.; soluble Zulkowski starch from Merck, Darmstadt, Germany.

**Isolation of pancreatic acini.** Pancreatic acini were isolated from pancreas of guinea pigs under halothane anesthesia according to the collagenase digestion procedure detailed by Peikin et al. [6] with a slight modification. HEPES-buffered Ringer’s solution (HBR: HEPES, 24.5 mM; NaCl, 98 mM; KCl, 6 mM; Na\(_2\)PO\(_4\), 2.5 mM; sodium pyruvate, 5 mM; sodium fumarate, 5 mM; sodium glutamate, 5 mM; glucose, 11.5 mM; L-glutamine, 2 mM; CaCl\(_2\), 2 mM; MgCl\(_2\), 1 mM; MEM, 1%; vitamin solution, 1%; BSA, 0.2%; TI, 0.01%; pH 7.4) (5 ml) containing purified collagenase (65–75 U/ml) was injected intramurally into isolated pancreas, and the

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pancreas was incubated at 37°C for 60 min with continuous shaking (120 cycles/ min). After the incubation, the pancreatic tissue was triturated five times with a glass pipette (2-mm tip diameter) and filtered through a nylon mesh (300-μm lattice). Isolated acini thus obtained were washed three times with enzyme-free HBR and used for measurements of amylase release or \([Ca^{2+}]_c\).

**Perfusion.** Isolated acini were resuspended in perfusion solution (HBR containing 0.5 mM CaCl₂ and 5 mM theophylline), transferred to a perfusion chamber consisting of a filter (5 μm lattice: Millipore Corp., Bedford, MA, U.S.A.) and a filter holder (Millipore Corp.), and perfused at a rate of 1 ml/min with a peristaltic pump. The effluent from the filter holder was collected every 2 or 5 min, and stored on ice for determination of amylase content.

**Measurement of amylase release.** Amylase activity was assayed by a modified method of Bernfeld using soluble Zulkowsky starch [18] and was expressed as a percentage of the total amylase activity contained in acini. To minimize the error due to seasonal variation in pancreatic responses to various agents, each set of experiments was performed together with the control.

**Measurement of \([Ca^{2+}]_c\).** Isolated acini were loaded with 2.5 μM Fura-2-AM (40 min, 37°C) and transferred to a perfusion chamber (1.5 ml). The ratio between fluorescence intensities at 510 nm with excitation at 340 and 380 nm was measured from single cells in pancreatic acini according to the method described by Kanno et al. [19]. \([Ca^{2+}]_c\) was calculated from the ratio according to the equation of Grynkiewicz et al. [20]. Pancreatic acini were perfused at the rate of 2 ml/min and drugs were added to the perfusion solution. The half-time for a complete change of the solution in the chamber was about 40 s.

**Statistics.** Results are expressed as the mean±S.E. of several experiments, and were analyzed by Student's t-test.

**RESULTS**

Potentiating effect of VIP on CCK-induced amylase release has already been reported in a wide range of CCK concentrations (30 pM–1 nM) in pancreatic acini of the guinea pig [6]. In the present study, we chose 100 pM CCK-8 and combined it with 30 pM–10 nM VIP to study the potentiation by these peptides.

**Potentiation of CCK-8-induced amylase release by VIP (30 pM–10 nM) or forskolin**

Figure 1 shows the effect of VIP (10 nM) on CCK-8 (100 pM)-induced amylase release. CCK-8 and VIP each evoked significant amylase release (Fig. 1A) and the peak amylase release reached 2.4- and 1.9-fold of the basal value, respectively. When CCK-8 and VIP were combined, the peak amylase release reached 5.5-fold of the basal value, which was significantly greater than the sum of amylase release induced by CCK-8 and VIP alone (3.3-fold of the basal value) (Fig. 1B).

The effects of VIP at lower concentrations (30 or 100 pM) on CCK-8 (100 pM)-induced amylase release were examined (Fig. 2). The combination of CCK-
Fig. 1. Effects of VIP on CCK-8-induced amylase release. A: Time courses of amylase release in response to CCK-8 alone (a), and to VIP alone (b), and that of unstimulated release (c). B: The time course of amylase release in response to the combination of CCK-8 and VIP (d) and that of the sum of amylase release in response to CCK-8 alone and VIP alone (e; calculated by a + b − c). Horizontal bars in this and subsequent figures indicate the time during which drugs were added. The values are means ± SE of 4 experiments. *p < 0.05, **p < 0.01, ***p < 0.001 compared between d and e.

CCK-8 (100 pm) and VIP (100 pm) induced greater amylase release (11.4-fold of the basal value) than the sum of amylase release induced by CCK-8 and VIP (100 pm) alone (5.9- and 2.4-fold of the basal value, respectively) (Fig. 2A). Although VIP (30 pm) alone did not induce significant amylase release, the combination of CCK-8 and VIP (30 pm) induced greater amylase release (9.3-fold of the basal value) than that induced by CCK-8 alone (5.9-fold of the basal value) (Fig. 2B).

To know whether the potentiating effect of VIP on CCK-8-induced amylase release is mediated by cAMP, the effect of forskolin (10 μM), an activator of

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Fig. 2. Effects of lower concentrations of VIP on CCK-8-induced amylase release. A: Time courses of amylase release in response to the combination of CCK-8 and VIP (100 pM) (a), to CCK-8 alone (b), and to VIP (100 pM) alone (c). B: Time courses of amylase release in response to the combination of CCK-8 and VIP (30 pM) (d), to CCK-8 alone (e), and to VIP (30 pM) alone (f). The values are means ± SE of 4 experiments. *p < 0.05, **p < 0.01, ***p < 0.001 compared between d and e.

adenylate cyclase, on CCK-8 (100 pM)-induced amylase release was measured. Figure 3A shows the time course of amylase release induced by CCK-8 alone or forskolin alone. Forskolin alone, as has been observed with VIP, induced significantly greater amylase release than the basal values. Figure 3B shows the time course of amylase release induced by the combination of CCK-8 and forskolin. The effect of forskolin was similar to that of VIP; the combination of CCK-8 and
Fig. 3. Effects of forskolin on CCK-8-induced amylase release. A: Time courses of amylase release in response to CCK-8 alone (a) and to forskolin alone (b), and that of unstimulated release (c). B: The time course of amylase release in response to the combination of CCK-8 and forskolin (d), and that of the sum of amylase release in response to CCK-8 alone and forskolin alone (e; see Fig. 1 legend). The values are means±SE of 5 experiments. *p<0.05, **p<0.01 compared between d and e.

forskolin induced significantly greater amylase release than the sum of the release induced by CCK-8 and forskolin alone. This result suggests that the potentiation of CCK-8-induced amylase release by VIP is mediated by cAMP as other known cellular events accompanied by VIP receptor activation including amylase release induced by VIP alone in pancreatic acinar cells [8].

Effects of VIP on CCK-8 induced $[Ca^{2+}]_c$ response

To elucidate the mechanism of VIP to potentiate CCK-8-induced amylase release...
release, the effects on \([\text{Ca}^{2+}]_c\) of VIP, CCK-8, and their combination were studied, since \([\text{Ca}^{2+}]_c\) is known as a mediator of CCK-8-induced response. Figure 4 shows the effect of VIP (10 nM) on the CCK-8 (100 pM)-induced \([\text{Ca}^{2+}]_c\) response. VIP alone did not affect \([\text{Ca}^{2+}]_c\) (data not shown), whereas CCK-8 alone induced Ca\(^{2+}\) oscillations (Fig. 4). In a few experiments, CCK-8 induced a transient increase in \([\text{Ca}^{2+}]_c\) (data not shown). The pattern of Ca\(^{2+}\) oscillations was fairly constant during the continuous stimulation with CCK-8 up to 20 min (data not shown). When VIP was added on top of CCK-8, the frequency or the amplitude, or both of CCK-8-induced Ca\(^{2+}\) oscillations were reduced (Fig. 4). Similar results were obtained with a lower concentration of VIP (100 pM) (data not shown).

This inhibitory effect of VIP on CCK-8-induced Ca\(^{2+}\) oscillations was quantitatively analyzed by measuring 1) the mean \([\text{Ca}^{2+}]_c\) rise from the baseline \((\Delta[\text{Ca}^{2+}]_c)\), 2) the Ca\(^{2+}\) spike amplitude, and 3) the time intervals between Ca\(^{2+}\) spikes before and after VIP addition (Fig. 5A–C). The \(\Delta[\text{Ca}^{2+}]_c\) and the amplitude were significantly decreased and the interval was significantly increased by VIP (10 nM).

**Effects of caffeine on CCK-8-induced Ca\(^{2+}\) oscillations and amylase release**

To test whether inhibition of Ca\(^{2+}\) oscillations can be advantageous in potentiating amylase release, we examined effects of caffeine (20 mM), which is known to inhibit CCK- or ACh-induced Ca\(^{2+}\) increase in mouse pancreatic acinar cells, on CCK-8 (100 pM)-induced amylase release and Ca\(^{2+}\) oscillations (Fig. 6). In pancreatic acini of the guinea pig, the addition of caffeine during the continuous stimulation with CCK-8 significantly inhibited CCK-8-induced amylase release (Fig. 6A) and reduced the frequency and the amplitude of CCK-8-induced Ca\(^{2+}\) oscillations (Fig. 6B).
Fig. 5. Effects of VIP (10 nM) on CCK-8 (100 pM)-induced Ca^{2+} oscillations. A: Comparison between the mean [Ca^{2+}]_c rise from the baseline (\Delta [Ca^{2+}]_c) before and after VIP addition. Each value was calculated from [Ca^{2+}]_c recorded for 4 min. B: Comparison between the amplitude of [Ca^{2+}]_c spikes just before VIP addition, and of the first (1) and second (2) spikes after VIP addition. C: Comparison between the interval between [Ca^{2+}]_c spikes just before VIP addition, and the first (1) and second (2) intervals after VIP addition. The values are means±SE of 9 experiments. *p<0.05, ***p<0.001 compared with CCK-8 alone.

Effects of low and high [Ca^{2+}]_c conditions on VIP-induced amylase release

To determine whether VIP can induce amylase release independently of [Ca^{2+}]_c, the effect of VIP (10 nM) was measured when [Ca^{2+}]_c was raised and lowered by thapsigargin (TG) and BAPTA, respectively. The combination of VIP (10 nM) and TG (10 μM) induced significantly greater amylase release than that
induced by VIP alone (Fig. 7A), whereas TG alone did not cause significant increase in amylase release (data not shown). TG caused a transient increase in $[Ca^{2+}]_c$ (Fig. 7B), which was unaffected by further addition of VIP (data not shown). When acini were pretreated with BAPTA-AM (25 $\mu$M) for 40 min and then perfused with a $Ca^{2+}$-free solution containing EGTA (1 mM) for 40 min, $[Ca^{2+}]_c$ decreased from the basal value of 182 ± 5 (n = 22) to 70 ± 9 nM (n = 4) (data not shown). This $[Ca^{2+}]_c$-lowering treatment abolished VIP-induced amylase release (Fig. 8). When $[Ca^{2+}]_c$ was restored amylase release rebounded...
Fig. 7. Effects of TG on VIP-induced amylase release and the TG-induced \([\text{Ca}^{2+}]_c\) increase. A: Time courses of amylase release in response to the combination of TG and VIP (a), and to VIP alone (b). B: The time course of \([\text{Ca}^{2+}]_c\) in response to TG alone. The values of A are means±SE of 3 experiments. *\(p<0.05\), **\(p<0.01\) compared between a and b.

transiently, indicating that the secretory machinery was not damaged during the \([\text{Ca}^{2+}]_c\)-lowering treatment. VIP-induced amylase release was inhibited but not abolished by pretreatment with BAPTA-AM alone or by perfusion with a \(\text{Ca}^{2+}\)-free solution alone (data not shown).

The relation between amylase release and \([\text{Ca}^{2+}]_c\) in normal, low and high \([\text{Ca}^{2+}]_c\) conditions is summarized in Fig. 9. VIP (10 nM)-induced amylase release increased as \([\text{Ca}^{2+}]_c\) increased, and the ratio of amylase release over \([\text{Ca}^{2+}]_c\) was similar in the \([\text{Ca}^{2+}]_c\) range tested in the present study. However, the ratio was
Fig. 8. Effects of Ca\(^{2+}\) depletion on VIP-induced amylase release. The time course of amylase release in response to VIP with (b) or without (a) pretreatment with BAPTA-AM (25 μM). In the group pretreated with BAPTA-AM, VIP was added in the absence of extracellular Ca\(^{2+}\). The values are expressed as means±SE of 3 experiments. *p < 0.05, **p < 0.01, ***p < 0.001 compared between a and b.

Fig. 9. Dependence of VIP-induced amylase release on [Ca\(^{2+}\)]_e. The amounts of amylase released with each treatment for 15 min in the presence or absence of VIP are shown. The numbers below the bars indicate averaged [Ca\(^{2+}\)]_e values obtained with the treatments without VIP (the averaged [Ca\(^{2+}\)]_e values obtained with CCK and VIP is also shown in the CCK treatment). The data are calculated from the results shown in Figs. 1, 4, 7, 8. The values are expressed as means±SE of 3–26 experiments. **p < 0.01, ***p < 0.001 compared with the values obtained without VIP. ⋆p < 0.05, ⋆⋆p < 0.01 compared with the values in the normal [Ca\(^{2+}\)]_e condition.

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clearly higher when acini were stimulated with the combination of CCK-8 and VIP: the combination caused a smaller \([\text{Ca}^{2+}]_c\) increase than TG, even though the combination caused much greater amylase release than the combination of TG and VIP.

**DISCUSSION**

Our main focus in the present study was to elucidate the mechanism underlying the potentiation of pancreatic enzyme secretion evoked by the combination of CCK and VIP that seem to act independently. The first possibility we examined was whether VIP potentiates CCK-induced \(\text{Ca}^{2+}\) oscillations, since it has been shown that cAMP potentiates \(\text{Ca}^{2+}\)-dependent cellular functions by amplifying \(\text{Ca}^{2+}\) entry or \(\text{Ca}^{2+}\) release from intracellular \(\text{Ca}^{2+}\) stores in various types of cells [21–23]. In the present study, we observed that VIP caused clear potentiation of CCK-induced amylase release without amplifying CCK-induced \(\text{Ca}^{2+}\) oscillations in pancreatic acinar cells of the guinea pig. On the contrary, our study has revealed that VIP unexpectedly suppresses CCK-induced \(\text{Ca}^{2+}\) oscillations.

There are two interpretations for these paradoxical effects of VIP on secretion and \(\text{Ca}^{2+}\) oscillations: a) the inhibition of \(\text{Ca}^{2+}\) oscillations is advantageous for pancreatic enzyme secretion and the inhibition per se accounts for the potentiation of amylase release or b) the potentiation by VIP occurs at a site other than the increase in \([\text{Ca}^{2+}]_c\) (namely, synthesis, or downstream to the increase in \([\text{Ca}^{2+}]_c\), or independently of \([\text{Ca}^{2+}]_c\)).

To examine the former possibility, we have studied the effect on amylase release of 20 mM caffeine, which is reported to inhibit \(\text{Ca}^{2+}\) oscillations in mouse pancreatic acinar cells [24]. In the pancreatic acini of the guinea pig, caffeine inhibited both amylase secretion and cytosolic \(\text{Ca}^{2+}\) oscillations, suggesting that the former possibility is unlikely.

In the latter possibility, it is also unlikely that VIP acts in the synthetic process, namely, stimulates synthesis of the pancreatic enzymes and their storage in the zymogen granules, since, in the present study, the stimulatory effect of VIP became evident within 5 min, whereas it is reported that labeling of the zymogen granule fraction appeared 57 min after incubation of guinea pig pancreatic slices with radioactive amino acids [25]. Thus, the key question is whether VIP can induce secretion in the absence of \(\text{Ca}^{2+}\), or whether it enhances some process downstream from the increase in \([\text{Ca}^{2+}]_c\). To test this, we have examined how the secretory response to VIP is influenced by changing \([\text{Ca}^{2+}]_c\). VIP-induced amylase release was potentiated when \([\text{Ca}^{2+}]_c\) was raised by TG, which is consistent with the previous finding that detectable potentiation of VIP-stimulated amylase secretion occurred with TG in rat pancreas [10]. On the other hand VIP-induced amylase release was completely abolished when \([\text{Ca}^{2+}]_c\) was lowered by pretreatment with BAPTA. These results indicate that VIP is not able to induce secretion, when \([\text{Ca}^{2+}]_c\) is as low as 70 nM. This is the first report that cAMP-mediated enzyme

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secretion requires a certain level of $[\text{Ca}^{2+}]_c$ in intact pancreatic acinar cells. In permeabilized pancreatic acini of the rat, it has been shown that cAMP-induced enzyme secretion is totally abolished when $[\text{Ca}^{2+}]_c$ was lowered to 10 nm [26]. VIP might increase Ca$^{2+}$ sensitivity of the exocytotic apparatus, since some phosphoproteins that are known to be involved in exocytosis have cAMP-dependent phosphorylation sites [27], or have strong homology with the catalytic subunit of cAMP-dependent protein kinase [28].

We conclude that VIP potentiates CCK-induced amylase release by facilitating a Ca$^{2+}$-dependent process distal to the increase in $[\text{Ca}^{2+}]_c$. However, further study is required to specify the site of VIP-induced potentiation of secretion.

The present study has revealed that VIP, in spite of its potentiating action on amylase release, inhibits CCK-induced Ca$^{2+}$ oscillations in the guinea pig pancreatic acini. One possible explanation for this inhibitory action of VIP is that VIP increased Ca$^{2+}$-pumping out to the extracellular space or Ca$^{2+}$ reuptake into intracellular Ca$^{2+}$ stores, thereby inhibited CCK-induced Ca$^{2+}$ oscillations, since it is reported that the catalytic subunit of cAMP-dependent protein kinase stimulates Ca$^{2+}$ pump in the plasma membrane of guinea pig pancreatic acinar cells [29] and that cAMP modulates Ca$^{2+}$ release from and uptake into ER [30–33]. The latter possibility is more likely since, in the present study, VIP did not reduce $[\text{Ca}^{2+}]_c$ when Ca$^{2+}$ pump in ER was inhibited by TG.

In the present study, TG alone caused a transient increase in $[\text{Ca}^{2+}]_c$, but did not induce significant amylase release. It could be that factors other than a rise in $[\text{Ca}^{2+}]_c$ are mandatory to evoke secretion effectively, or that TG raised $[\text{Ca}^{2+}]_c$ at a region remote from the exocytotic region. On the other hand, in the rat pancreas, TG was reported to cause a significant increase not only in $[\text{Ca}^{2+}]_c$ but also in amylase release [10, 34].

The present study confirmed previous reports that VIP potentiates CCK-induced amylase release [4–6, 15, 16], and moreover, showed that the potentiation became evident about 5 min after VIP addition and this was maintained throughout VIP stimulation. The potentiation was observed with VIP at a concentration as low as 30 pm, which by itself had no effect on amylase release. Considering these results together with the facts that mammalian pancreatic tissues possess abundant VIP-containing neurons [1] and that the concentration of VIP measured at the portal vein after vagal stimulation in pigs was about 30 pm [35, 36], the present results strengthen the view that VIP plays a key role in pancreatic enzyme secretion.

In summary, VIP stimulates pancreatic enzyme secretion without causing an increase in $[\text{Ca}^{2+}]_c$, however, the stimulation of secretion critically depends on $[\text{Ca}^{2+}]_c$. Moreover, VIP potentiates CCK-induced secretion while inhibiting CCK-induced Ca$^{2+}$ increase. This may be advantageous for maintaining secretion at a high rate without inducing various harmful effects of Ca$^{2+}$ which have been suggested to occur when $[\text{Ca}^{2+}]_c$ is elevated above a certain level [37, 38].

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