Supramaximal CCK and CCh concentrations abolish VIP potentiation by inhibiting adenylyl cyclase activity

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Akiyama, Toshiharu, Yoshihide Hirohata, Yoshinori Okabayashi, Issei Imoto, and Makoto Otsuki. Supramaximal CCK and CCh concentrations abolish VIP potentiation by inhibiting adenylyl cyclase activity. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G1202–G1208, 1998.—Exocrine pancreatic secretion stimulated by vasoactive intestinal polypeptide (VIP), which acts through the adenylyl cyclase-cAMP pathway, is potentiated by stimulation with other secretagogues such as CCK and carbachol (CCh). However, the potentiating effect is abolished by the same secretagogues at supramaximal concentrations. In the present study, we examined the mechanisms by which supramaximal concentrations of CCK octapeptide (CCK-8) or CCh reduce the VIP-induced potentiation of amylase secretion from isolated rat pancreatic acini. VIP-stimulated amylase secretion was potentiated by submaximal stimulatory concentrations of CCK-8 and CCh but was reduced by the same reagents at higher concentrations. Supramaximal concentrations of CCK-8 or CCh also reduced forskolin-induced potentiation of amylase release but did not reduce that induced by 8-bromo-cAMP. Moreover, supramaximal concentrations of CCK-8 or CCh inhibited VIP-stimulated intracellular cAMP production as well as adenylyl cyclase activity. 12-O-tetradecanoylphorbol 13-acetate (TPA) also reduced the magnitude of the potentiation of amylase release caused by VIP plus CCK-8 or CCh, although TPA itself decreased neither VIP-stimulated adenylyl cyclase activity nor intracellular cAMP accumulation. These results indicate that supramaximal concentrations of CCK-8 and CCh reduce the potentiating effect of VIP and forskolin on amylase secretion by inhibiting the adenylyl cyclase activity. In addition, protein kinase C is suggested to be partly implicated in this inhibitory mechanism. The mechanisms that lead to such inhibition may be interlinked but distinct from each other.

isolated pancreatic acini; adenosine 3',5'-cyclic monophosphate; protein kinase C

THE EXOCRINE PANCREATIC function is regulated by a number of hormones and neurotransmitters, which have their respective receptors on the acinar cells and stimulate enzyme secretion through activating the intermediary mechanisms coupled with their receptors. Two major intermediary pathways have been identified; one is the adenylyl cyclase-cAMP system and the other involves a receptor-mediated stimulation of hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) to inositol 1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG) (29). It has been reported that secretagogues that act through the adenylyl cyclase-cAMP pathway, such as secretin and vasoactive intestinal polypeptide (VIP), potentiate the pancreatic secretion in response to those that act through the phosphoinositides pathway, such as CCK, carbachol (CCh), and bombesin (5, 8, 22). The increase caused by the two secretagogues in combination was greater than the sum of the increase caused by each secretagogue acting alone (potentiation). In addition to this synergistic effect, it is known that supramaximal concentrations of CCK octapeptide (CCK-8) progressively reduce the magnitude of potentiation of enzyme secretion that occurs with VIP plus CCK-8 (5, 8, 22, 29). However, the mechanism of this CCK-induced restricted potentiation of pancreatic enzyme secretion has not yet been clarified. Previous studies have reported controversial results. Collen et al. (5) have postulated that secretagogues that mobilize cellular Ca2+ modulate the action of VIP on enzyme secretion at postreceptor loci, because these secretagogues do not alter binding of 125I-VIP or intracellular cAMP or the increase in intracellular cAMP accumulation caused by VIP. On the other hand, Katsushima et al. (13) and Murakami et al. (19) have demonstrated that CCK and CCh decrease the specific binding of 125I-VIP, VIP-induced intracellular cAMP accumulation, and VIP-stimulated amylase secretion.

The present study was performed to investigate extensively the mechanisms by which supramaximal concentrations of CCK-8 and CCh for stimulating amylase secretion reduce VIP-induced potentiation in isolated rat pancreatic acini.

MATERIALS AND METHODS

Chemicals. We purchased the following chemicals: soybean trypsin inhibitor type 1-S (SBTI), CCh, pilocarpine, 12-O-tetradecanoylphorbol 13-acetate (TPA), 8-bromo-cAMP (8-Br-cAMP), forskolin, IBMX, HEPES, and phenylmethylsulfonyl fluoride (PMSF) from Sigma Chemical (St. Louis, MO); chromatographically purified collagenase (type CLSPA) from Cooper Biochemical (Malvern, PA); minimal Eagle’s medium amino acid supplement from Gibco Laboratories-LifeTechnologies (Grand Island, NY); BSA (fraction V) from Armour Pharmaceutical (Phoenix, AZ); and Phadebas amylase test (amylase test A) from Shionogi Pharmaceutical (Osaka, Japan); CCK-8, synthetic porcine VIP, and bombesin from Peptide Institute (Protein Research Foundation, Osaka, Japan); CCK-derived peptide (MV-180) from Research Plus (Bayonne, NJ); CAMP RIA kit from Yamasa Shoyu (Chiba, Japan); and [α-32P]ATP (370 MBq/ml) from Amersham International (Buckinghamshire, UK).
Preparation of isolated pancreatic acini. Isolated rat pancreatic acini were prepared from male Wistar rats (weighing 250 g and fed ad libitum) by the method reported previously (21). The basic medium used to prepare the isolated acini was modified Krebs-Henseleit bicarbonate buffer (KHB), containing 1.1 mM glucose, 0.1 mg/ml SBTI, and minimal Eagle’s medium amino acid supplement and was gassed with 95% O2-5% CO2. The preparation and incubation medium was HEPES-buffered Ringer solution (HR). HR was similar to KHB but contained 10 mM HEPES (pH 7.4) as buffer and 5 mM BSA and was gassed with 100% O2.

Amylase release. In all experiments, acini were allowed to recover in HR for 30 min at 37°C as a baseline at a density of 1.0–1.5 mg acinar protein/ml while being shaken 60 times/min. After recovery, acini were centrifuged and resuspended in fresh HR at a density of 0.3–0.4 mg acinar protein/ml. Two-milliliter aliquots were distributed into 25-ml polycarbonate flasks. The amylase-releasing effects of receptor-mediated secretagogues, such as CCK-8, CCh, bombesin, pilocarpine, and J MV-180, and receptor-bypassing agents, such as forskolin, 8-BrcAMP, and TPA, were determined similarly in the presence or absence of 10 nM VIP.

Amylase release into the extracellular medium during a 30-min incubation at 37°C with various secretagogues was expressed as a percentage of the total content of the enzyme present in the acinar pellet at the beginning of the incubation period (21). Amylase activity was measured using Phadebas amylase test A (3). In all experiments, at least duplicate, mostly triplicate flasks were used to determine the amylase release stimulated by each concentration of secretagogues.

Intracellular cAMP content. cAMP content in the acini was measured according to a previously reported method (10). Acini were incubated with various concentrations of CCK-8, CCh, bombesin, JMV-180, pilocarpine, and TPA with or without 10 nM VIP in the presence of 1 mM IBMX for 30 min at 37°C. At the end of the incubation period, 1 ml of sample was centrifuged at 3,000 g for 10 s. The sediment was homogenized by ultrasonic disintegration (200 kHz, 10 s). 1 ml of 0.1 N HCl was added, and cAMP and protein concentrations were determined. The cAMP assay was carried out using a Yamasa cAMP RIA kit. Cellular cAMP was calculated relative to the protein concentration.

Adenylyl cyclase activity in membrane preparation. Adenylyl cyclase activity was measured in acinar membrane preparations, according to the general method of Salomon et al. (24). Briefly, acini were pelleted by centrifugation (600 g, 10 min), after which acini were homogenized by ultrasonic disintegration (200 kHz, 10 s) in homogenization buffer (50 mM Tris-HCl, pH 7.5, 3 mM EDTA, 0.1 mM PMSF, and 5 µg/ml SBTI). Homogenate was centrifuged at 27,000 g for 10 min. The pellet was washed once in homogenization buffer and resuspended. The standard incubation mixture contained 50 mM Tris-HCl, pH 7.5, 5 mM 2-mercaptoethanol, 1.5 mM EDTA, 0.05 mM PMSF, and 2.5 µg/ml SBTI, 5 mM MgCl2, 150 nM cAMP, 10 mM ATP, and [α-32P]JATP (0.2–5 mCi/tube), 20 mM creatinine phosphate, 100 U/ml of creatine phosphokinase, 5 mM theophylline, and 50–100 u of membrane protein in a total volume of 200 µl. cAMP production, as a measurement of adenylyl cyclase catalytic activity, was measured at 37°C for 10 min as described previously (24).

Data analysis. Results were expressed as means ± SE. Statistical analysis was performed using Student’s t-test for unpaired samples. Differences with P < 0.05 were considered to be statistically significant.

RESULTS

CCK-8 and CCh had biphasic dose-response curves for stimulating amylase release in that, as the secretagogue concentrations increased, amylase release increased, became maximal, and then decreased with supramaximal concentrations (Fig. 1, A and B). To examine the potentiating interactions between VIP and CCK-8 or CCh in stimulating amylase release, we compared the dose-response curve obtained with CCK-8 or CCh alone with that obtained in the presence of a fixed concentration of VIP (10 nM). The configuration of the dose-response curves for CCK-8 and CCh in combination with VIP was the same with as it was without VIP. With 10 nM VIP, adding CCK-8 up to 100 pM caused progressively greater potentiation of VIP-stimulated enzyme secretion, whereas with concentrations of CCK-8 > 100 pM, there was a progressive decrease in the magnitude of potentiation, and with 10 nM CCK-8 the value for amylase secretion with VIP was nearly the same as that without VIP (Fig. 1A). The pattern of the potentiation caused by the simultaneous addition of various concentrations of CCh to 10 nM VIP (Fig. 1B) was similar to that caused by the simultaneous addition of CCK-8 and VIP (Fig. 1A). CCh at concentrations of 0.1–10 µM caused potentiation of VIP-stimulated amylase release, whereas CCh at >100 µM abolished VIP-induced potentiation (Fig. 1B).

On the other hand, the dose-response curve caused by bombesin was monophasic, in that supramaximal concentrations of the peptide (>1 nM) did not cause submaximal stimulation of amylase secretion (Fig. 1C). With 10 nM VIP, adding bombesin up to 1 nM caused progressively greater potentiation of amylase secretion. In contrast to CCK-8 and CCh, however, there was no decrease in the magnitude of potentiation even with concentrations of bombesin >1 nM (Fig. 1C). Consistent with previous studies (11, 15, 16), dose-response curves for amylase release caused by JMV-180, a partial agonist of CCK-A receptor (16), and pilocarpine, a partial agonist at cholinergic receptors (11), were monophasic. Enzyme secretion was maximal with 10 nM JMV-180 (Fig. 2A) and 10 µM pilocarpine (Fig. 2B). With increasing concentrations of JMV-180 up to 10 nM or pilocarpine up to 10 µM, there was a progressively greater potentiation of VIP-stimulated amylase secretion. Supramaximal concentrations of these secretagogues, similar to bombesin, did not reduce VIP-induced potentiation of amylase secretion (Fig. 2).

Because the secretory function of VIP is mediated through adenylyl cyclase-cAMP systems, we then examined the effect of forskolin and 8-BrcAMP on potentiation of amylase secretion in response to CCK-8 and CCh. First, we compared the dose-response curve obtained with CCK-8 or CCh alone with that obtained in the presence of a fixed concentration of forskolin, which directly activates adenylyl cyclase, increases intracellular levels of cAMP, and stimulates amylase secretion (6). With 10 µM forskolin, adding CCK-8 up to 100 µM (Fig. 3A) or CCh up to 3 µM (Fig. 3B) caused progressively greater potentiation of amylase secretion,
whereas with supramaximal concentrations of these secretagogues (CCK-8 > 100 pM; CCh > 3 µM), there was a progressive decrease in the magnitude of potentiation (Fig. 3).

This result suggests that the reduction of potentiation is caused by alterations in adenylyl cyclase and/or its downstream molecules. We then compared the dose-response curve obtained with CCK-8 or CCh alone with that obtained in the presence of a fixed concentration of 8-BrcAMP, a membrane-permeable analog of cAMP. With 10 µM 8-BrcAMP, adding CCK-8 up to 100 pM or CCh up to 3 µM caused progressively greater potentiation of 8-BrcAMP-stimulated amylase secretion (Fig. 4). In contrast to VIP (Fig. 1) and forskolin (Fig. 3), there was no decrease in the magnitude of potentiation with concentrations of CCK-8 above 100 pM or CCh above 3 µM (Fig. 4). These results raise the possibility that supramaximal concentrations of CCK-8 or CCh
work through decreasing VIP-induced intracellular cAMP accumulation and thereby reducing the magnitude of the VIP-induced potentiation of amylase secretion.

We then examined the interaction between VIP and various secretagogues in stimulating intracellular cAMP production. Supramaximal concentrations of CCK-8 (>100 pM) and CCh (>3 µM) significantly and to a similar extent suppressed the VIP-stimulated increase in intracellular cAMP concentration, although CCK-8 itself increased intracellular cAMP levels at higher concentrations (Table 1). In contrast, bombesin, JMV-180, and pilocarpine did not alter the increase in intracellular cAMP accumulation caused by 10 nM VIP (Table 1).

To confirm that supramaximal concentrations of CCK-8 and CCh work through inhibiting VIP-stimulated adenylyl cyclase activity, we then measured mem-
Methods, after treatment with either VIP alone or together with various secretagogues in isolated rat pancreatic acini, VIP-stimulated adenylyl cyclase activity in rat pancreatic acinar membrane was measured. Membranes from rat pancreatic acini were prepared and adenylyl cyclase activity was measured in the presence of 1 mM IBMX, TPA, 12-O-tetradecanoylphorbol 13-acetate (TPA), carbachol, forskolin, or forskolin. *P < 0.05 vs. control (none). † P < 0.05 vs. lower concentrations of CCK-8 or CCh. ‡ P < 0.05 vs. 10 nM VIP alone.

These results indicate that supramaximal concentrations of CCK-8 and CCh reduce VIP-induced potentiation of amylase secretion by inhibiting adenylyl cyclase activity. Supramaximal concentrations of CCK-8 and CCh seem to induce excessive protein kinase C (PKC) stimulation compared with bombesin, J MV-180, and pilocarpine. To address whether PKC is involved in the mechanisms by which supramaximal concentrations of CCK-8 or CCh reduce the VIP-induced potentiation of amylase secretion in response to 10 nM VIP, we examined the effect of the phorbol ester TPA on amylase secretion in response to VIP in combination with CCK-8 or CCh. TPA greatly decreased the magnitude of potentiation caused by simultaneous addition of VIP and CCK-8 or CCh (Table 3).

We then examined the effect of TPA on VIP-stimulated intracellular cAMP accumulation and adenylyl cyclase activity. As shown in Tables 1 and 2, however, TPA itself had no influence on VIP-induced cAMP accumulation and adenylyl cyclase activity. These results are consistent with the findings that amylase secretion in response to 0.01–1 µM TPA was not inhibited but rather potentiated in the presence of 10 nM VIP (data not shown).

Discussion

Consistent with previous reports (2, 5, 20, 25), there was a progressive potentiation of VIP-stimulated amylase secretion with increasing concentrations of CCK-8 or CCh up to 100 pM or 3 µM, respectively, whereas increasing the concentration of these secretagogues up to supramaximal concentrations reduced the magnitude of VIP-induced potentiation of amylase secretion. In the present study, we have shown that the reduction of this potentiation is due to the inhibition of VIP-stimulated adenylyl cyclase activity by supramaximal concentrations of CCK-8 or CCh.

Previous studies (5, 6) have demonstrated that CCK-8 and CCh decrease the specific binding of 125I-VIP to rat pancreatic acini, suggesting that the reduction of potentiation by supramaximal concentrations of CCK-8 and CCh is caused by the inhibition of the binding of VIP to its receptor. However, that would not explain our present observation that supramaximal concentrations of CCK-8 or CCh decrease the magnitude of potentiation induced by forskolin, which directly activates adenylyl cyclase, increases intracellular cAMP accumulation, and stimulates amylase secretion by bypassing receptors (6). It is possible, therefore, that supramaximal concentrations of CCK-8 or CCh might have caused submaximal potentiation caused by VIP not only at the receptor sites but also at the postreceptor loci. Although supramaximal concentrations of CCK-8 and CCh decreased VIP-stimulated adenylyl cyclase activity in membrane preparations and intracellular cAMP accumulation, they did not reduce the maximal potentiation induced by 8-BrcAMP, a membrane-permeable analog of cAMP. On the other hand, bombesin, pilocarpine, and TPA inhibited but rather potentiated in the presence of 10 nM VIP, as shown in Tables 1 and 2.
J MV-180 neither altered the VIP-stimulated adenyl cyclase activity and intracellular cAMP accumulation nor reduced VIP-stimulated potentiation of amylase release. Taken together, these present observations suggest that supramaximal concentrations of CCK-8 and CCh reduced the magnitude of the potentiation by inhibiting the adenyl cyclase activity and intracellular cAMP accumulation.

With respect to the mechanisms through which supramaximal concentrations of CCK-8 and CCh reduced the adenyl cyclase activity and intracellular cAMP accumulation, it is conceivable that PKC might be implicated, since PKC has been shown to alter adenyl cyclase function in some systems (1, 4, 9, 12, 14, 18, 23, 27). The primary event engendered by CCK or muscarinic receptor occupancy in pancreatic acini is an increased hydrolysis of PIP2 in the plasma membrane by an associated phospholipase C to produce DAG, which activates PKC, and IP3, which increases intracellular Ca2+ concentration ([Ca2+]i) (28, 29). Stimulation of amylase secretion by bombesin, as with CCK and cholinergic agonists, is also dependent on the hydrolysis of PIP2 with the production of IP3 and DAG (1, 28). CCK-8 and bombesin are equally efficacious in stimulation of amylase secretion, whereas the magnitude of an increase in acinar DAG in response to bombesin was much smaller than the response to CCK-8 (17). J MV-180 also induces maximal amylase release similar to CCK-8, but it increases DAG only to the same extent as bombesin (15). Similarly, pilocarpine increases phosphoinositide turnover but to a much lesser extent compared with CCh (30). The smaller increase in DAG induced by bombesin, J MV-180, and pilocarpine compared with CCK-8 or CCh could be interpreted as indicating that excessive PKC stimulation is involved in the inhibition of VIP-stimulated adenyl cyclase activity and potentiation of amylase release. Indeed, bombesin, J MV-180, and pilocarpine neither inhibited the VIP-stimulated intracellular cAMP accumulation nor the VIP-induced potentiation of amylase secretion. Several reports (1, 4, 9, 12, 14, 18, 23, 27) have shown the regulation of adenyl cyclase by PKC. PKC is shown to inhibit adenyl cyclase activity stimulated by some agonists, including adrenergic agonists, glucagon, parathyroid hormone, and histamine (9, 12, 14, 18, 23). In addition, in HT-29 cells, preincubation with TPA has been shown to decrease maximum VIP-stimulated adenyl cyclase activity as well as the number of cell surface VIP receptors (27). In contrast, some reports (1, 4) have shown that PKC stimulates hormone-responsive adenyl cyclase. In the present study, the phorbol ester TPA decreased the magnitude of the potentiation of amylase release caused by simultaneous addition of VIP and CCK-8 or CCh. This indicates that PKC might be implicated in the inhibition of VIP-induced adenyl cyclase activation by supramaximal concentrations of CCK-8 and CCh. However, the PKC inhibitor staurosporine (1 mM) failed to restore the reduction of VIP-induced potentiation of amylase release stimulated by supramaximal concentrations of CCK-8 and CCh (unpublished observation). This may result from almost complete inhibition of PKC by staurosporine. In addition, TPA itself decreased neither VIP-stimulated adenyl cyclase activity in membrane preparation nor intracellular cAMP accumulation as has been reported by Suwandito et al. (26). This finding is consistent with our findings that VIP caused potentiation of amylase release in response to TPA. Therefore, our results suggest that PKC itself is not sufficient and factors other than excessive PKC stimulation are necessary to reduce the VIP-induced potentiation of amylase secretion by supramaximal concentrations of CCK-8 and CCh.

With respect to [Ca2+]i, it is unlikely that the pattern of Ca2+ signaling is involved in the inhibition of VIP-induced potentiation of amylase release. Higher concentrations of CCK-8 (>100 pM), CCh, and bombesin (1–10 nM) are shown to induce a large transient increase in [Ca2+]i, followed by a small sustained increase in [Ca2+]i, although low concentrations of these secretagogues induce repetitive oscillations in [Ca2+]i (15, 17). In contrast, stimulation with J MV-180 and pilocarpine at all concentrations has been reported only to result in the generation of an oscillatory [Ca2+]i signal (15, 16). Thus the pattern of Ca2+ signaling of bombesin is similar to CCK-8 and CCh but different from that of J MV-180 and pilocarpine, whereas, unlike CCK-8 and CCh, higher concentrations of bombesin as well as J MV-180 and pilocarpine neither decreased amylase secretion nor reduced VIP-induced potentiation of enzyme secretion.

Calmodulin is shown to be implicated in PKC modulation of prostacyclin-activated adenyl cyclase (25). We examined the effect of the calmodulin kinase inhibitor N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide (W-7) (100 µM) on amylase release in response to 10 nM VIP in combination with supramaximal concentrations of CCK-8 and CCh and found that W-7 had no effect on it (unpublished observations). Studies (unpublished observations) with the tyrosine kinase inhibitor genistein (300 µM) indicate that tyrosine kinase is not implicated in this mechanism. On the other hand, it is suggested, in Swiss 3T3 fibroblasts, that CCK activation of phospholipase C-mediated hydrolysis of phosphatidylincholine plays an important role in the mechanism whereby muscarinic agonists inhibit adenyl cyclase (7). Further studies are needed to clarify the precise mechanisms by which supramaximal concentrations of CCK-8 and CCh reduce adenyl cyclase activity in pancreatic acini.

In conclusion, our present results indicate that supramaximal stimulatory concentrations of CCK-8 and CCh decrease the magnitude of the potentiating effect of VIP and forskolin on amylase secretion by inhibiting adenyl cyclase activity. In addition, PKC is suggested to be partly implicated in this inhibitory mechanism. The mechanisms that lead to such inhibition may be interlinked but distinct from each other.

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