Vol. 164, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 16, 1989 Pages 8-13

INOSITOL TRISPHOSPHATE INDEPENDENT INCREASE OF INTRACELLULAR FREE CALCIUM AND AMYLASE SECRETION IN PANCREATIC ACINI

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Received August 2, 1989

It is generally believed that the activation of various cell surface receptors results in the phospholipase C-catalyzed production of inositol trisphosphate which, in turn, increases the intracellular concentration of free Ca^{2+} by stimulating its release from non-mitochondrial sources. We have investigated both the production of inositol trisphosphate and changes in intracellular Ca^{2+} concentration in rat pancreatic acini in response to caerulein and CCK-JMV-180, two analogs of cholecystokinin. Both of these analogs cause comparable increases in the rate of amylase secretion and in intracellular Ca^{2+} concentration but their effects on inositol phosphate generation are dramatically different; caerulein stimulates significant production of inositol phosphates within 1 min of its addition, whereas no detectable levels of inositol phosphates were generated within the same time after addition of CCK-JMV-180. These results suggest that the CCK-JMV-180 stimulated release of intracellular Ca^{2+} is not mediated by inositol trisphosphate but some other as yet unidentified messenger.

Secretion of digestive enzymes from pancreatic acinar cells is regulated by hormones and neurotransmitters which bind to receptors located on the cell surface. During the past decade, considerable progress has been made in elucidating the sequence of events known as stimulus-secretion coupling (1). These events link receptor occupancy by secretagogues to the secretion in pancreatic acinar cells and many other types of secretory cells. It is generally accepted that occupancy of a specific receptor by the gut hormone cholecystokinin (CCK) or the neurotransmitter acetylcholine results in the activation of the membrane-bound enzyme phospholipase C, which then hydrolyses the membrane lipid phosphatidylinositol bisphosphate releasing inositol trisphosphate (IP_3) and diacylglycerol

0006-291X/89 $1.50

(2,3). IP_3 is believed to increase the intracellular concentration of free calcium ions ($Ca^{2+}i$) by stimulating its release from non-mitochondrial sources, possibly, the recently discovered organelles, known as calciosomes (4-6). $Ca^{2+}i$ and diacylglycerol, in turn, stimulate secretion by mechanisms that are not well understood but which probably involve some type of kinase activity.

In the present study we have investigated the mechanism of stimulus-secretion coupling in rat acini by examining the production of inositol phosphates and changes in $Ca^{2+}i$ concentration in response to caerulein, a decapeptide analog of CCK, and in response to CCK-JMV-180, a recently developed synthetic analog of CCK with the structure Boc-Tyr(So_3)Nle-Gly-Trp-Nle-Asp-2-phenylester (7,8). These studies indicate that CCK-JMV stimulates amylase secretion and causes the release of intracellular calcium but it does not cause a demonstrable stimulation in IP_3 production.

METHODS AND MATERIALS

Male Wistar rats were obtained from Charles River Breeding Laboratories. [^{3}H]-myoinositol was purchased from Amersham. Collagenase and soybean trypsin inhibitor were obtained from Worthington Biochemicals. Caerulein was purchased from Peninsula Labs. CCK-JMV-180 was purchased from Research Plus, Bayonne, N.J. All other chemicals were purchased from Sigma Chemical Co.

Dispersed pancreatic acini from rat pancreas were prepared by collagenase digestion and gentle shearing as described earlier (9). Freshly prepared acini were suspended in 20 ml of N-2-hydroxyethylpiperizine-N^1-2-ethanyl sulfonic acid (HEPES)-Ringer buffer (pH=7.4) containing 118 mM NaCl, 5 mM KCl,1 mM $MgCl_2$, 0.1% bovine serum albumin, 1.26 mM $CaCl_2$, 0.01% soyabean trypsin inhibitor, and 1% Eagle's basal amino acids and 10 mM HEPES. The buffer was saturated by bubbling with 100% of O_2. After 20 min of preincubation acini were washed with this buffer and resuspended in the same buffer containing various concentrations of either caerulein or CCK-JMV-180. Amylase secretion was measured over 30 min at 37° C. α-amylase activity was measured using soluble starch as substrate according to the method of Bernfeld (10).

For the measurement of inositol phosphates, acini were labelled by incubating with myo-[^{3}H]-inositol (100 uCi/ml) for 120 min at 37° C in HEPES-Ringer buffer. After washing with the same buffer, these labelled acini were incubated with or without secretagogues. The stimulation was stopped after 1 min by the addition of cold 0.4 N (final concentration) perchloric acid. The samples were allowed to precipitate in ice for 30 min and then centrifuged (300xg for 15min). Supernatants were adjusted to pH 8.5 by the addition of a neutralization buffer (0.5 N KOH, 9 mM $Na_2B_4O_7$ and 1.9 mM EDTA) and inositol phosphates were separated by ion-exchange chromatography on Dowex (11) and eluted fractions were counted in scintiverse II. Results of inositol phosphate generation are expressed as percent of control counts, which were obtained when acini were incubated without any secretagogues.

Intracellular free calcium was monitored by using the fluorescent Ca^{2+} indicator, fura-2. Briefly, acini were incubated with 2 uM acetoxymethylester of fura-2 for 30 min

at 37° C. The cells were washed and resuspended in HEPES-Ringer buffer. Fluorescence (excitation at 340 and 380 nm; emission at 505 nm) was monitored with a SPEX dual excitation spectrofluorimeter. Intracellular calcium concentration was calculated as described by Grynkyevicz et al (12).

RESULTS

Amylase secretion: The effect of caerulein and CCK-JMV-180 on amylase secretion from dispersed pancreatic acini is shown in Figure 1. In agreement with many previous studies, caerulein caused a dose-dependent stimulation of amylase secretion with maximal rate of net secretion (11.9% of the total/30 min) observed in the presence of 10^{-10}M caerulein. Higher doses of caerulein resulted in lower rates of amylase secretion. CCK-JMV-180, like caerulein also caused a dose-dependent stimulation of amylase release from pancreatic acinar cells, with the maximal rate secretion (12.2% of total/30 min,$p > .05$ compare to caerulein) obtained with 10^{-7}M CCK-JMV-180. However, in contrast to caerulein higher concentrations of CCK-JMV-180 did not decrease the rate of amylase secretion.

Intracellular calcium changes: Both caerulein and CCK-JMV-180 caused a dose-dependent rise in the intracellular Ca^{2+} concentration with maximal increase (above resting $Ca^{2+}i$ levels) of 366 $\pm$ 40 nM obtained with 10^{-9} caerulein and 345 $\pm$ 45 nM with 10^{-6} CCK-JMV-180 (Figure 2). Chelation of extracellular calcium by addition of 4 mM EGTA to the

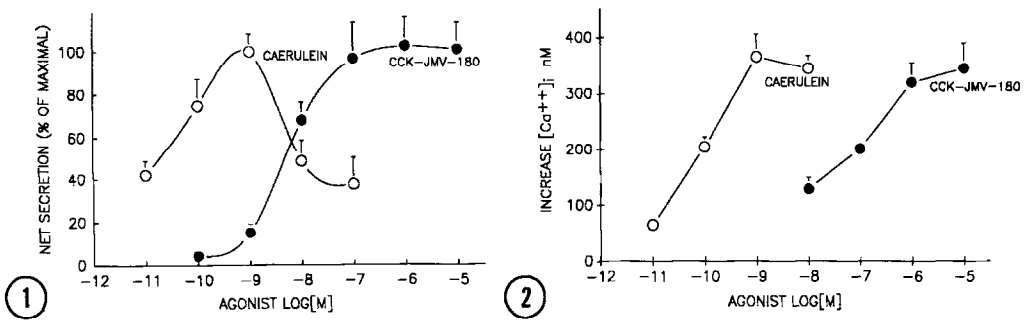

Figure 1. Effect of CCK-JMV-180 and caerulein on amylase secretion. Rat pancreatic acini were incubated for 30 min with various concentrations of either CCK-JMV-180 (●—●) or caerulein (○—○) and the amount of amylase secreted in to the medium was quantititated. The results are expressed as percent of maximal secretion (obtained with 10^{-10}M caerulein, 11.9% of total). Results shown are mean values obtained from at least 5 different experiments.

Figure 2. Intracellular calcium concentration measurements. Rat acini were loaded with fura-2 and fluorescence changes were monitored following stimulation with various concentrations of CCK-JMV-180 (●—●) or caerulein (○—○). The fluorescence intensity was converted into calcium concentration as described in the text. The results are expressed as maximal increase (stimulated minus basal) in calcium concentration following stimulation. Results shown are mean $\pm$ SEM obtained from more than six separate acini preparations.

TABLE 1
Effect of EGTA on intracellular calcium mobilization

	Intracellular Calcium [nM]	
	Basal	Stimulated
Caerulein Alone	182 ± 25	548 ± 35
Caerulein + EGTA	160 ± 20	532 ± 45
CCK-JMV-180 alone	190 ± 18	525 ± 31
CCK-JMV-180 + EGTA	178 + 22	525 + 48

Rat pancreatic acini were loaded with fura-2 and calcium concentration was determined as described in the text. EGTA (4 mM) was added before the stimulation with caerulein (10^{-9}M) or CCK-JMV-180 (10^{-5}M). The results shown are means ± SEM obtained from four different experiments.

incubation medium did not alter the increase in intracellular calcium concentration induced by either caerulein or CCK-JMV-180 (Table 1).

Inositol phosphate formation: As shown in Figure 3, caerulein caused a dose-dependent stimulation of ^{3}H-inositol phosphates (IP, IP_2 and IP_3). Caerulein-stimulation of inositol phosphate production was detected within 1 min of its addition. In contrast, no increase in inositol phosphate formation was detected within 1 min of CCK-JMV-180 addition.

DISCUSSION

These results indicate that caerulein and CCK-JMV-180, two analogs of CCK, cause comparable increases in the rate of amylase secretion and rise in $Ca^{2+}i$ concentration in pancreatic acini. On the other hand, the effects of these two ligands on inositol phosphate generation are dramatically different. Caerulein stimulates production of IP, IP_2 and IP_3 within 1 min of its addition while, after CCK-JMV-180 addition, no increase in IP, IP_2 and IP_3 generation can be detected.

The dose-response relationship for amylase secretion by caerulein is biphasic with stimulation at lower concentrations and inhibition at higher concentrations. This has been variously interpreted to indicate that caerulein either interacts with two classes of receptors (high affinity stimulatory receptor and lower affinity inhibitory receptor) or that interaction of high concentrations of caerulein with a single class of receptors down-regulates the response(1). The newly developed analog CCK-JMV-180 has lower potency for both amylase secretion and increasing $Ca^{2+}i$ concentration than caerulein (Fig 1) but it does not cause high-dose inhibition. This finding suggest that CCK-JMV-180 either interacts as an agonist only at the high affinity stimulatory receptors or that it is incapable of inducing down regulation.

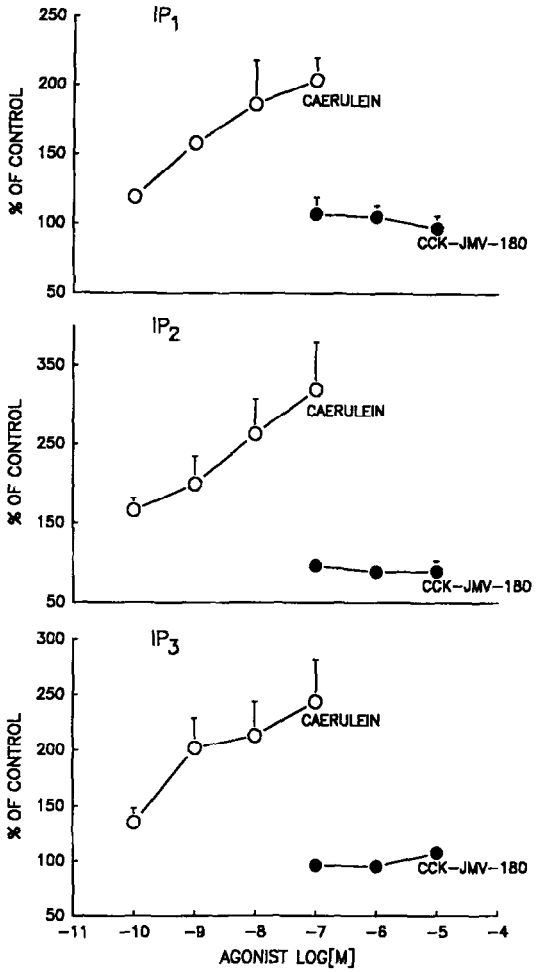

Figure 3. Production of inositol phosphates in rat pancreatic acini. Levels of ^{3}H-inositol phosphates in acini after one min of incubation with various doses of CCK-JMV-180 (●—●) or caerulein (○—○) were measured as described in the text. Results shown are mean ± SEM for 4 to 6 separate experiments for each ligand concentration.

The increase in Ca^{2+}i which follows addition of caerulein to acini is believed to reflect Ca^{2+} release from intracellular stores (4). It appears that CCK-JMV-180 also causes release of Ca^{2+} from intracellular stores since chelation of extracellular Ca^{2+} by EGTA did not reduce the rise in Ca^{2+}i which occured after addition of either caerulein or CCK-JMV-180. In the case of caerulein, release of Ca^{2+} from intracellular stores is generally accepted to be mediated by IP_3 generated from membrane phosphatidylinositol bisphosphate by phospholipase C (3). Indeed, we have observed that caerulein stimulates IP_3 production. In contrast to caerulein, the increase in Ca^{2+}i concentration which followed addition of CCK-JMV-180 was not associated with a rise in IP_3 production. Several explanations for this surprising observation are possible. For example, CCK-JMV-180 stimulated Ca^{2+} mobilization might be mediated by a very small amount of IP_3 which

is present in amounts which escape detection by currently available techniques. A corollary of this hypothesis would be that much of the IP_3 generated by caeruein is irrelevant to Ca^{2+} mobilization. An alternative explanation should also be considered - i.e. that the mediator of Ca2+ release stimulated by CCK-JMV-180 is not IP_3 but some other as yet unidentified messenger. If so, it is tempting to speculate that the same agent mediates caerulein stimulated Ca2+ mobilization and that, in the case of both CCK analogs, that agent is released in response to high affinity receptor occupancy. Thus, the generation of IP_3 by caerulein may reflect either low affinity receptor occupancy or down regulation of the high affinity system.

ACKNOWLEDGMENTS

We would like to thank Dr. Roberto Bruzzone for many helpful discussions and Ms. Anne Zavertnik and Mr. Wayne Glover for their superb technical assistance. This work was supported in part by NIH grant #AM31914.

REFERENCES

1. Williams, J.A., and Hootman, S.R. (1986) In The Exocrine Pancreas (Go, V.L.W., Ed.) pp. 123-139. Raven Press, N.Y.

2. Berridge, M.J. (1983) Biochem. J. 212, 849-858.

3. Berridge, M.J. (1988) In Phosphoinositides and Receptor Mechanisms (Putney, J.W.,Jr., Ed.) pp. 25-46, Alan R. Liss, N.Y.

4. Putney, J.W., Jr., Takemura, H., Hughes, A.R., Horstman, D.A., and Thastrup, O. (1989) FASEB Journal 3, 1899-1905.

5. Streb, H., Irvine, R.F., Berridge, M.J. and Schulz, I. (1983) Nature (London) 306, 67-68.

6. Volpe, P., Krause, K.H., Hashimoto, S., Zorzato, F., Pozzan, T. and Meldolesi, J. (1988) Proc. Nat. Acad. Sci. USA 85,1091-1095.

7. Galas, M.C. Lignon, P.F. Rodriguez, M., Mendie, C. Fulcrad, P. Lave, J., and Martinez, J. (1988) Am. J. Physiol. 254, G176-182.

8. Gaisano, H.Y., Klueppeiberg, N.G., Pinon, D.I., Pfenning, M.A., Powers, S.P.,and Miller, L.J. (1989) J. Clin. Invest. 83,321-325.

9. Saluja, A., Saluja, M., Powers, R.E., Houlihan, M.J., and Steer, M.L. (1986) Am. J. Physiol. 250, 413-417.

10. Bernfeld, P. (1955) In Methods in Enzymology (S.P.Colowick and N.D. Kaplan, Eds.) 1, pp. 149, Academic Press, N.Y.

11. Powers, R.E., Saluja, A.K., Houlihan, M.J., and Steer, M.L. (1985) Biochem. Biophys. Res. Commun. 131, 284-288.

12. Grynkyevicz, G., Poenie, M., and Tsien, R.Y. (1985) J. Biol Chem. 260, 3440-3450.