BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **235,** 197–200 (1997)
ARTICLE NO. RC976762

# Inhibitory Action of Sphingosine or Ceramide on Amylase Secretion from Isolated Rat Pancreatic Acini

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Received May 6, 1997

**Sphingosine and ceramide, products of sphingomyelin hydrosis by sphingomyelinase, have recently been regarded as second messengers for cell biological actions. On the other hand, exocrine pancreas is a typical organ to perform regulatory secretion of digestive enzymes, depending upon extracellular signals. We investigated the effects of sphingosine or ceramide on amylase secretion from isolated rat pancreatic acini. Either sphingosine or cell-permeable ceramide inhibits CCK8- or carbachol-induced enzyme secretion from isolated rat pancreatic acini in a dose-dependent manner. Sphingosine or ceramide itself does not affect basal amylase secretion from the acini. Ceramide also inhibits NaF-induced amylase secretion, indicating that it acts post the activation of receptor-linked GTP-binding protein. In our experiments, ceramide inhibited $Ca^{2+}$ ionophore-induced amylase secretion, but not phorbol ester-induced secretion. These results indicate that ceramide affects secretory processes post intracellular $Ca^{2+}$ mobilization in the exocrine pancreas.** 

Recently sphingomyelin hydrolysis has been recognized as an important pathway of intracellular signal transduction. The metabolic products of this pathway, such as ceramide, sphingosine, and sphingosine-1-phosphate have been reported to be involved into intracellular signal transduction systems for physiological or pathophysiological events (1-9). For instance, biologic effects of 1 $\alpha$, 25-dihydroxyvitamin $D_3$, tumor necrosis factor-$\alpha$, interferon-$\gamma$, and interleukin-1 correlate with early activation of neutral sphingomyelinase and elevation of ceramide in target cells(1) (2). In addition, the accumulated evidences that cell-permeable ceramides can elicit the biological actions of these extracellular stimuli in target cells nominate ceramide as a second messenger(2) (10) (11). Similarly, sphingosine has been also reported as a second messenger for cell proliferation of cultured fibroblasts from several origins and human tumor cell(12) (13).

Exocrine pancreas is a typical organ to perform regulatory secretion of digestive enzymes, depending upon extracellular signals, such as cholecystokinin or acetylcholine, in physiologic condition. In pancreatic acini, sphingosine has been reported to induce an oscillatory increase in the cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) (14), and to inhibit $Ca^{2+}$ uptake into the agonist-sensitive pool as well as inhibiting microsomal $Ca^{2+}$-ATPase(15). However, the effects of sphingosine or ceramide on enzyme secretion from exocrine pancreas have not been reported.

In this communication, we demonstrate their inhibitory effects on amylase secretion from isolated rat pancreatic acini and discuss molecular mechanism and significance of this phenomenon.

## MATERIALS AND METHODS

*Materials and chemicals.* Male Wistar rats (200-250 g wt) were employed. CCK8 (sulfate form) was purchased from Peptide Institute Inc., Osaka, Japan. Soybean trypsin inhibitor and carbachol were from Sigma Chemical Co., St. Louis, MO, U.S.A. D-erythro-sphingosine, N-Acetyl (C2 ceramide), D-erythrosphingosine, Dihydro-, N-Acetyl (C2 dihydroceramide), D-erythrosphingosine, free base (sphingosine) were obtained from Calbiochem-Novabiochem Co., LA Jolla, U.S.A.. 4 bromo-A23187 was from Nacalai Tesque, Inc., Kyoto, Japan. Purified collagenase was from Worthington Biochemicals (Freehold, NJ, U.S.A.); minimal Eagle's medium amino acid was from Gibco (Grand Island, NY); bovine serum albumin (fraction V) was from Armour Pharmaceutical Co., Tarrytown, NY, U.S.A.. Other materials and chemicals were obtained from commercial sources.

*In vitro amylase secretion.* Isolated pancreatic acini were prepared from a male Wistar rat by collagenase digestion and forceful pipetting as described previously(16). Freshly prepared acini were resuspended in an appropriate volume of Hepes-buffered Ringer's solution (Ringer's solution containing 10 mM Hepes at pH 7.4, 0.1

This investigation was supported in part by Grant-in-Aids from the Ministry of Education, Science, and Culture of Japan, 1996.

Abbreviations used: CCK8, cholecystokinin octapeptide; NaF, sodium fluoride; Hepes, N-3-hydroxyl-ethyl-piperazine-N-2-ethanesulfonic acid; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.



0006-291X/97 $25.00

mg/ml of soybean trypsin inhibitor, 5 mg/ml of bovine serum albumin, 11.1 mM glucose, minimal Eagle's medium amino acid supplement, and 1.25 mM $CaCl_2$) that was gassed with oxygen before use at a density of 2-3 mg/ml of protein in a 50-ml polycarbonate Erlenmeyer flask and preincubated for 30 min at 37°C. After the preincubation, the acini were resuspended in an appropriate volume of the same fresh solution at a density of 0.5 mg/ml of protein. Two-ml aliquots of the acinar suspension were distributed into 25-ml polycarbonate Erlenmeyer flasks, gassed with oxygen for 20 s, and incubated for 10 min at 37°C in the presence or absence of C2 ceramide, C2 dihydroceramide, or sphingosine and then incubated for 20 min at 37°C with secretagogues as indicated in each experiment. After the incubation, 1-ml aliquots were taken and immediately centrifuged at 10,000 g for 20 s. The amount of released amylase was expressed as the ratio of the value of amylase activity released into the medium during the incubation to that of total amylase content. The total amylase content was estimated by measureing the enzymatic activity of 1-ml aliquots of the unstimulated acinar suspension after disruption of the acinar cells by sonication.

## RESULTS

*Effect of C2 ceramide or C2 dihydroceramide on in vitro amylase secretion induced by CCK8.* The effect of C2 ceramide or C2 dihydroceramide on in vitro CCK8-induced amylase secretion from rat pancreatic acini was examined. As we previously reported (16), amylase secretion was stimulated in a dose-dependent manner at lower concentrations of CCK8, the maximal rate of amylase secretion was observed with $1 \times 10^{-10}$ M CCK8, while amylase secretion was inhibited at high concentrations than $1 \times 10^{-10}$ M CCK8. When 100 $\mu$M C2 ceramide was added to the acini before the incubation with CCK8, the dose-dependent curve moved below. In the presence of C2 ceramide, amylase secretion decreased to about 55% of in the absence of C2 ceramide, which had been observed with either $1 \times 10^{-10}$ M or $1 \times 10^{-8}$ M of CCK8 (Fig. 1A).

In contrast, when 100 $\mu$M C2 dihydroceramide was added to the acini before the incubation with CCK8, the dose-dependent curve did not move significantly. C2 dihydroceramide did not affect amylase secretion induced by CCK8 (Fig. 1B).

*Effect of C2 ceramide on in vitro amylase secretion induced by carbachol.* Incubation of the acini with carbachol, which is a cholinagic stimulant to exocrine pancreas, induced marked amylase secretion in a dose-dependent manner. The maximal and half maximal effects were observed with $3 \times 10^{-6}$ M and $3 \times 10^{-7}$ M of calbachol, respectively. When 100 $\mu$M C2 ceramide was added to the acini before the incubation with carbachol, the dose-dependent curve moved below. In the presence of C2 ceramide, amylase secretion decreased to about 75% of in the absence of C2 ceramide, which had been observed with maximal and submaximal dose of carbachol, respectively (Fig. 2).

*Dose effect of C2 ceramide or sphingosine on in vitro amylase secretion induced by CCK8.* Figure 3A shows

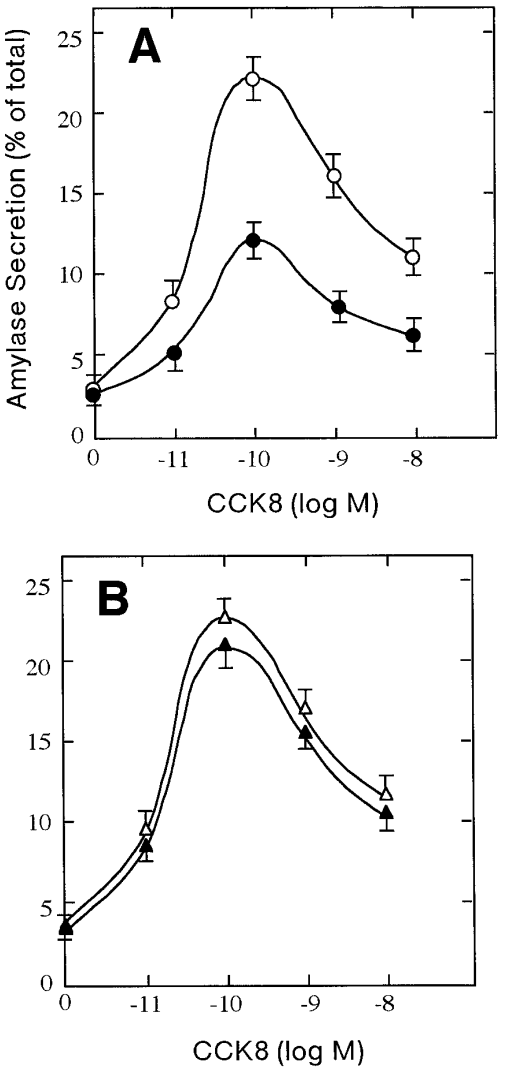


**FIG. 1.** Effect of C2 ceramide or C2 dihydroceramide on in vitro amylase secretion induced by CCK8. The acini were incubated for 10 min in the presence or absence of 100 $\mu$M C2 ceramide or C2 dihydroceramide and then incubated for 20 min at 37°C with indicated concentrations of CCK8. (A) In the absence (○) and presence (●) of C2 ceramide. (B) In the absence (△) and presence (▲) of C2 dihydroceramide. The results shown are representative of three independent experiments.

the dose-response of C2 ceramide on the amylase secretion elicited by maximal dose of CCK8. Increasing amounts of C2 ceramide inhibited progressively amylase secretion induced by $1 \times 10^{-10}$ M CCK8. In the presence of 100 $\mu$M C2 ceramide, amylase secretion was decreased to about 55% of in the absence of C2 ceramide. C2 ceramide alone in the amounts used in this experiment did not affect amylase secretion in the absence of CCK8. Figure 3B shows the dose-response of sphingosine on amylase secretion elicited by maximal dose of CCK8. Increasing amounts of sphingosine

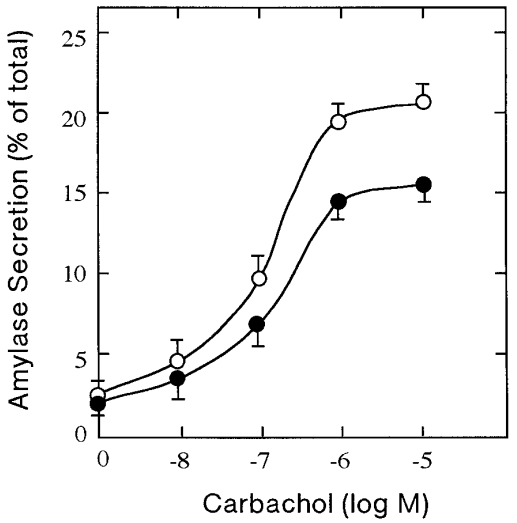


**FIG. 2.** Effect of C2 ceramide on in vitro amylase secretion induced by carbachol. The acini were incubated for 10 min in the presence or absence of C2 ceramide and then incubated for 20 min at 37°C with indicated concentrations of carbachol. (○) In the absence of C2 ceramide; (●) in the presence of C2 ceramide. The results shown are representative of three independent experiments.

as well as C2 ceramide inhibited progressively amylase secretion induced by $1 \times 10^{-10}$ M CCK8. In the presence of 40 $\mu$M sphingosine, amylase secretion was decreased to about 55% of in the absence of sphingosine. Sphingosine alone in the doses used in these sets of experiment did not affect amylase secretion in the absence of CCK8.

*Effect of C2 ceramide on amylase secretion induced by other secretagogues.* When 100 $\mu$M C2 ceramide was added to the acini before the incubation with 10 mM NaF, amylase secretion was inhibited to 78.1% of control (Table I). While C2 ceramide did not inhibit amylase secretion induced by 50 ng/ml TPA only, C2 ceramide inhibited the amylase secretion elicited by 2 $\mu$M A23187 only or 50 ng/ml TPA plus 2 $\mu$M A23187 to 75.4% and 77.3% of control, respectively (Table I).

## DISCUSSION

In this communication, we firstly demonstrated that sphingosine or cell-permeable ceramide inhibits CCK8- or carbachol-induced enzyme secretion from isolated rat pancreatic acini in a dose-dependent manner. Sphingosine or ceramide itself does not affect basal amylase secretion. Ceramide also inhibits NaF-induced amylase secretion, indicating that it acts post the activation of receptor-linked GTP-binding protein. Furthermore, ceramide inhibits $Ca^{2+}$ ionophore-induced amylase secretion, but not phorbol ester-induced one.

Concerning inhibitory action of sphingosine or ceramide on secretory process, Scita and Wolf reported inhibitory action of sphingosine (0.5-10 $\mu$M) on fibronectin secretion from human lung fibroblast(13). Nakamura *et al.* recently reported that C2 ceramide (30 $\mu$M) inhibit antigen-induced serotonin release to 5.9%, and A23187-induced serotonin release to 75.2% in rat basophilic leukemia (RBL-2H3) cells(11). The mode of action of ceramide in secretory process post to $Ca^{2+}$ mobilization in the cells seems similar to that in the rat pancreatic acini. From the results obtained here, we assume that sphingosine or ceramide at least inhibits

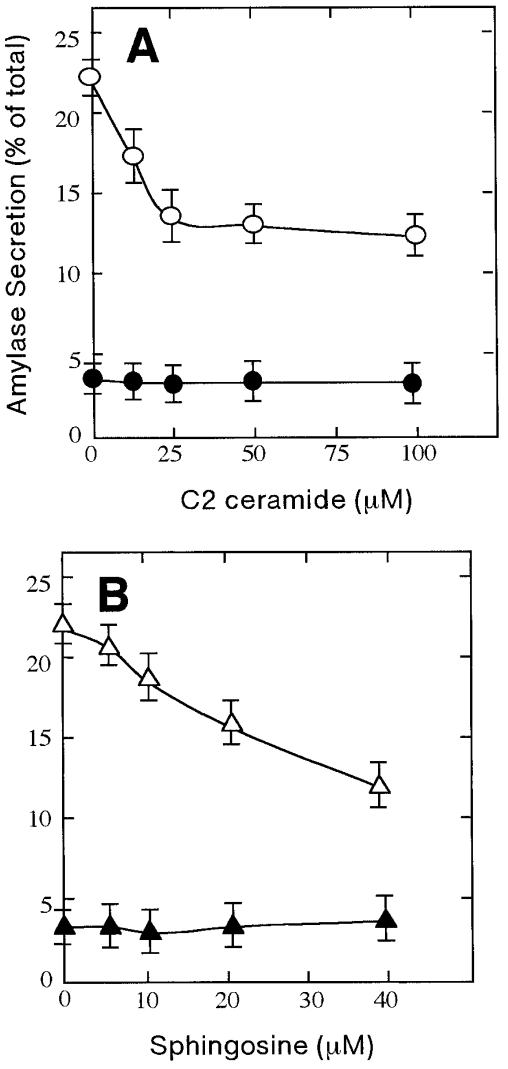


**FIG. 3.** Dose effect of C2 ceramide or sphingosine on in vitro amylase secretion induced by CCK8. The acini were incubated for 10 min in the presence of various amounts of C2 ceramide or sphingosine and then incubated for 20 min at 37°C in the presence of $1 \times 10^{-10}$ M CCK8 or absence of CCK8. (A) With C2 ceramide, in the presence (○) and absence (●) of CCK8. (B) With sphingosine, in the presence (△) and absence (▲) of CCK8. The results shown are representative of three independent experiments.

**TABLE I**

Effect of C2 Ceramide on Amylase Secretion Induced by Various Secretagogues[a]

| Secretagogues | Amylase secretion (% of total) (A) Control | (B) C2 ceramide | (B)/(A) (%) |
|---|---|---|---|
| None | 3.0 ± 0.5 | 2.9 ± 0.5 | 96.6 |
| NaF (10mM) | 16.9 ± 1.2 | 13.2 ± 0.9 | 78.1 |
| TPA (50ng/ml) | 13.2 ± 0.5 | 13.0 ± 0.4 | 98.4 |
| A23187 (2$\mu$M) | 11.0 ± 0.6 | 8.3 ± 0.4 | 75.4 |
| TPA (50ng/ml) + A23187 (2$\mu$M) | 30.5 ± 1.5 | 23.2 ± 1.2 | 77.3 |

[a] The acini were incubated for 10 min in the presence or absence of 100 $\mu$M C2 ceramide and then incubated for 20 min at 37°C with the indicated dose of various secretagogues, and amylase secretion was assayed as described in Methods. Values are expressed as percent of released amylase amount to total amylase content. Results are means ± SE of three independent experiments.

secretory process(es) post to $Ca^{2+}$ influx in exocrine pancreas.

The inhibition of enzyme secretion in exocrine pancreas can be associated with acute pancreatitis. When CCK, or its analogue caerulein, is administered to rat in a supraphysiologic dose, edmatous pancreatitis develops constantly with marked depression of exocrine secretion(17). The inhibition of enzyme secretion is derived from impairment of intracellular transport of zymogen granules, and can be reconstructed in vitro by supramaximal stimulation of CCK8 ($>1 \times 10^{-9}$ M), which is exhibited by biphasic dose response curve as illustrated in Fig. 1A. As the mode of secretory inhibition is thought to be a key to solve the onset machanism of acute pancreatitis, many investigators have focused on this matter.

In 1982, Sankaran *et al.* reported that there are two classes of CCK receptor with different affinities on rat pancreatic acini(18). They also proposed that the occupancy of the high affinity sites is related to the stimulation of amylase release, but the occupancy of the low affinity sites is related to the supression of amylase release. Since their proposal, many investigators have focused on the linkage of the low affinity sites with the inhibitory action of amylase release(19) (20). Although it has been clarified that there are at least 3 classes of receptors for CCK with different affinities(21), the molecular mechanism of secretory inhibition has not been elucidated.

We previously demonstrated that this inhibitory process is post to $Ca^{2+}$ mobilization and protein kinase C activation(22), and the microtubule disorganization in intracelular vesicular transport is involved in this secretory impairment(23). Although an idea is raised that sphingomyelin hydrolysis is involved in this mechanism, it is less possible because further secretory inhibition is achieved by sphingosine or C2 ceramide on impaired secretion elicited by supramaximal dose of CCK8 as shown in Fig. 1A. However, the possibility that sphingosine or its related metabolites are involved in the onset mechanism of acute pancreatitis as intracellular messenger can not be denied, and physiological or pathological stimuli inducing sphingomyelin hydrolysis in exocrine pancreas should be searched and investigated.

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