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Sphingosine regulates Ca^{2+}-ATPase and reloading of intracellular Ca^{2+} stores in the pancreatic acinar cell

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Abstract

The purpose of present study was to examine the effects of sphingosine on cellular Ca^{2+} transports using dispersed rat pancreatic acini. The results demonstrated that sphingosine had a specific effect to inhibit Ca^{2+} uptake into the cell's agonist-sensitive pool as well as inhibiting microsomal Ca^{2+}-ATPase. The ability of sphingosine to inhibit Ca^{2+} uptake resulted in both augmentation of Ca^{2+} release from the pool by inositol 1,4,5-trisphosphate (IP_3) and conversion of the Ca^{2+} release by inositol 1,4,5-trisphosphate from a transient response to a sustained response. Furthermore, by preventing Ca^{2+} pool refilling sphingosine mimicked the effect of the agonist, carbachol, to maintain an increased $[Ca^{2+}]_i$ during sustained stimulation. These results suggest that regulation of Ca^{2+}-ATPase by sphingosine or a sphingosine-like agent mediates some of the effects of agonist on cell Ca^{2+} transports.

Keywords: Sphingosine; Calcium; Acetylcholine; (Pancreas)

1. Introduction

In the pancreatic acinar cell, agonists such as cholecystokinin (CCK) and acetylcholine analogues cause a phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol [1–3]. IP_3 in turn, releases calcium from the intracellular agonist-sensitive pool into cytoplasm [4,5]. This release increases the cytoplasmic $[Ca^{2+}]$ ($[Ca^{2+}]_i$) [6]. The increase in $[Ca^{2+}]_i$ is largely transient because the release from the pool is a transient phenomenon [7,8] and because the cytoplasmic Ca^{2+} is pumped to cell exterior by plasma membrane Ca^{2+}-ATPases [9]. During sustained agonist stimulation the internal agonist-sensitive calcium pool remains partially calcium depleted [7,10]. Recent studies demonstrate that the depleted pool stimulates Ca^{2+} influx across the plasma membrane into the cytosol [11–15]. The mechanism connecting the pool and plasma membrane Ca^{2+} influx involves cyclic GMP [16]. The Ca^{2+} entering the cytosol increases $[Ca^{2+}]_i$ and is available for reloading the agonist-sensitive pool [12,16].

Recent reports demonstrated effects of sphingosine on cell Ca^{2+} transports. In one report [17] sphingosine caused both calcium release from and inhibited calcium reuptake into the intracellular Ca^{2+} pool of a smooth muscle cell line. In another report [18] sphingosine stimulated Ca^{2+} influx in rat parotid acinar cells. Sphingosine has been suggested to be an endogenous regulator of protein kinase C activity and to be released by sphingomyelinase action on sphingomyelin [19]. Because of these reported effects of sphingosine on cellular calcium, we questioned if sphingosine or a sphingosine-like substance could mediate the effects of agonists on cell calcium transports. In the present series of experiments, we demonstrated that sphingosine inhibited Ca^{2+} reloading of the cell's agonist-sensitive Ca^{2+} store. This action of sphingosine was due to its effect on the store Ca^{2+}-ATPase. The results of the experiments suggested that the sustained effect of the agonist on Ca^{2+} depletion of the store and Ca^{2+} influx

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across the plasma membrane were due to an inhibitory effect on store Ca^{2+}-ATPase activity. Sphingosine or a sphingosine-like substance may mediate this effect.

2. Materials and methods

2.1. Materials

Rats (150–200 g) were from Harlan Sprague Dawley. *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) and bovine serum albumin (fraction V) were from Boehringer-Mannheim. Soybean trypsin inhibitor, EGTA, carbachol, atropine, sphingosine, ouabain, were from Sigma. Oligomycin, 4 bromo-A23187, and inositol 1,4,5-trisphosphate were from Calbiochem. Purified collagenase (type CLSPA) was from Worthington. $^{45}CaCl_2$ (4–5 Ci/g calcium) was from Amersham. Thapsigargin was from L.C. Services. Fura-2/AM was from Molecular Probes (Junction City, OR). Synthetic COOH-terminal octapeptide of cholecystokinin (CCK-8) was a gift from Squibb Institute for Medical Research (Princeton, NJ).

2.2. Methods

Tissue preparation

Dispersed acini were prepared from rat pancreas using a collagenase digestion technique previously described [20,21].

Measurement of $[Ca^{2+}]_i$

Dispersed acini were loaded with fura-2/AM and $[Ca^{2+}]_i$ measured spectrofluorometrically as previously described [11]. For these experiments acini were incubated in incubation solution as previously described [10]. The quantity of $CaCl_2$ or EGTA in the incubation solution used for each experiment is described in the figure legends.

Measurement of $^{45}Ca^{2+}$ *uptake in permeabilized pancreatic acini*

Pancreatic acini were preincubated for 3 min with 100 μM carbachol in incubation solution containing no added $CaCl_2$ and 0.2 mM EGTA at 37°C in order to deplete the intracellular stores of Ca^{2+}. The action of carbachol was then terminated with the addition of 10 μM atropine. The acini were then washed and placed in a previously described [7] 'intracellular media' containing 120 mM KCl, 3 mM $MgCl_2$, 10 mM Hepes, 2 mM ATP, 10 μM antimycin, 5 μM oligomycin. The $^{45}Ca^{2+}$ was adjusted to 200 nM as previously described [7]. The acini were then electroporated and $^{45}Ca^{2+}$ uptake measured as previously described [7].

Preparation of rough microsomes and Ca^{2+}*-ATPase assay*

Pancreata from three rats (200–250 g) were used to prepare microsomes as described previous [22]. The

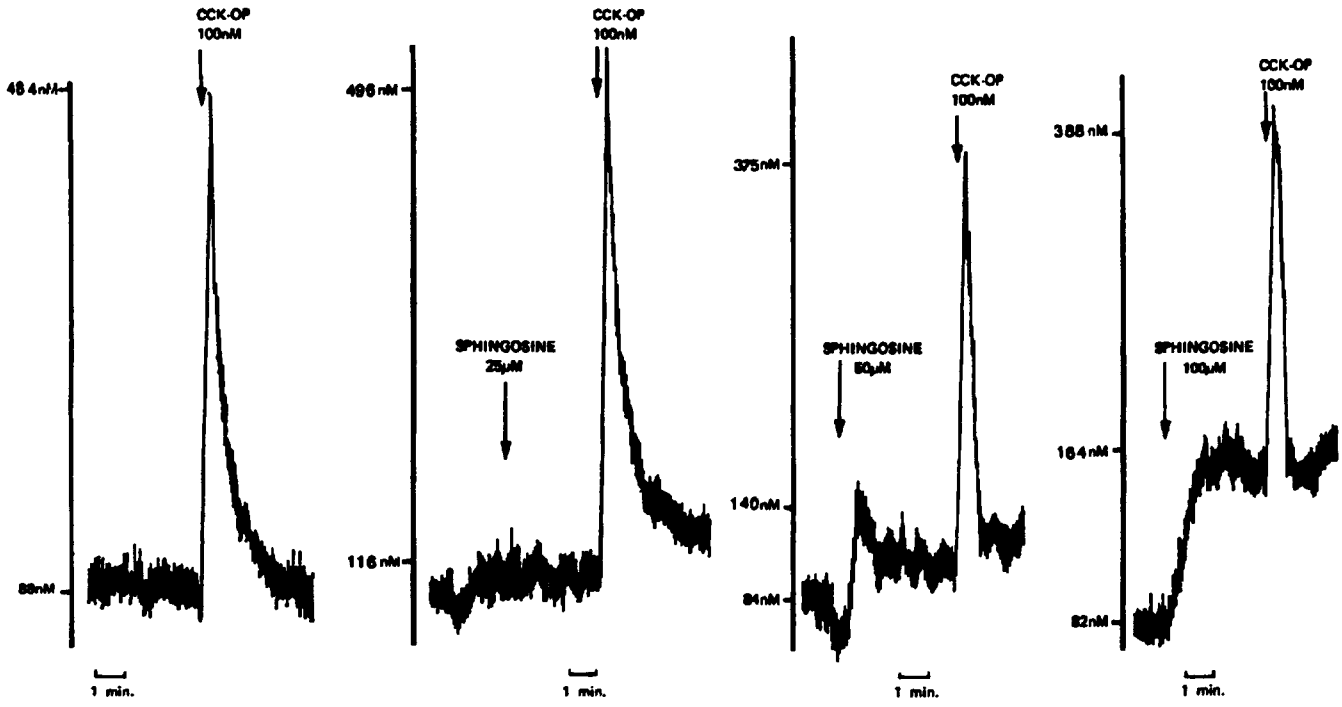

Fig. 1. The effect of sphingosine on intracellular calcium release. Dispersed acini loaded with fura-2/AM were transferred to incubation solution containing no added $CaCl_2$ and 0.2 mM EGTA just before the measurement and incubated at 37°C. Additions were made where indicated. These tracings are from a single experiment representative of four others.

Ca^{2+}-ATPase assay was adapted from a previously described procedure [23]. The rough microsome preparation was collected and diluted 3–4-fold (6 ml) in solution containing 20 mM Hepes (pH 7.0), 100 mM KCl, 5 mM $MgCl_2$, 1 mM ouabain and 1 μM 4 bromo-A23187. Aliquots of 250 μl were used for the Ca^{2+}-ATPase assay. Each aliquot contained 30 μg protein/μl). Each aliquot incubated with the indicated concentrations of thapsigargin, sphingosine or vehicle control for 20 min at 37°C. Each aliquot was then divided into two 100 μl fractions. One fraction was then incubated with 50 μM $CaCl_2$ while the other with 50 mM EGTA. The reaction was started by the addition of 1 mM ATP. The incubation was continued for 10 min. The reaction was stopped with the addition of 500 μl of 5% (w/v) trichloroacetic acid. Each sample was centrifuged at 3000 rpm in a Sorvall RT 6000 centrifuge for 10 min at 4°C. The supernatant inorganic phosphorus (P_i) was measured using a modification [24] of the method described by Fiske and SubbaRow [25]. Ca^{2+}-ATPase activity was determined in each aliquot as the P_i measured in the fraction with $CaCl_2$ added minus the P_i measured in the fraction with EGTA added. Ca^{2+}-ATPase activity for each aliquot was expressed as a percent of the activity measured in control aliquot (i.e., incubation with vehicle only).

3. Results and discussion

In the experiments illustrated in Fig. 1, we determined the effect of sphingosine on Ca^{2+} release using measurements of $[Ca^{2+}]_i$ in acini preloaded with fura-2/AM and incubated in media containing EGTA and no added $CaCl_2$. Thus, changes in $[Ca^{2+}]_i$ would result from changes in the partition of Ca^{2+} between that sequestered in internal stores and that free in the cytoplasm. The free form was measured as an increase in $[Ca^{2+}]_i$. Addition of sphingosine (25–100 μM) caused a dose-dependent increase in $[Ca^{2+}]_i$ suggesting that it resulted in translocation of calcium from the internal stores to the cytoplasm. 25 μM and 100 μM sphingosine increased $[Ca^{2+}]_i$ up to 1.42 ± 0.1 ($n = 4$) and 2.0 ± 0.005 ($n = 5$) times, respectively. EC_{50} for dose-response curve was 37.5 μM. Sphingosine at concentrations 20–100 μM did not produce any increase in LDH release as compared to control cell, suggesting that these doses of sphingosine are not toxic for pancreatic acinar cells.

The subsequent addition of a maximally effective concentration of CCK-octapeptide (CCK-OP) released all of the stored calcium resulting in a rapid large increase in $[Ca^{2+}]_i$ (Fig. 1). CCK-OP administered after the larger doses of sphingosine resulted in an attenuation of the effect of CCK-OP on $[Ca^{2+}]_i$

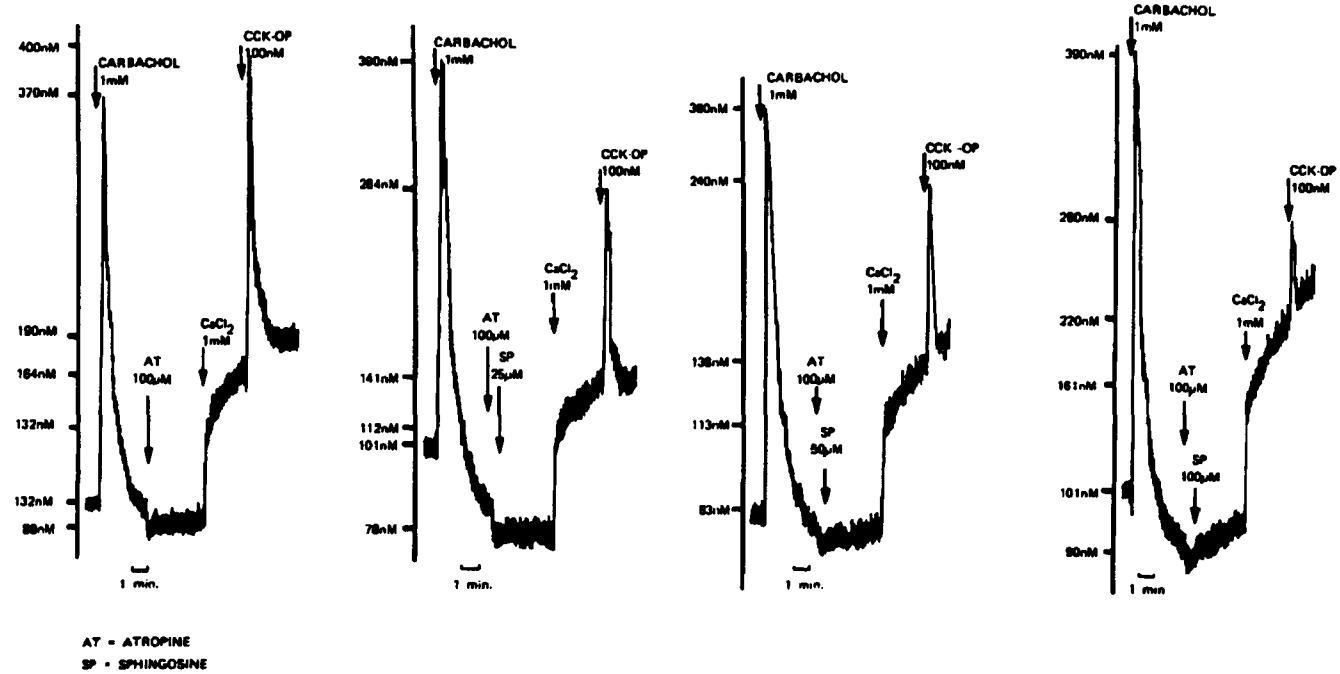

Fig. 2. Effect of sphingosine on calcium reloading of the intracellular pool. Fura-2/AM-loaded acini were transferred to standard incubation solution containing no added $CaCl_2$ and 0.2 mM EGTA just before measurement and incubated at 37°C. Additions were made where indicated. These are tracings of a single experiment representative of six others.

(Fig. 1). For example, 10^{-8}M CCK increased $[Ca^{2+}]_i$ in control cells by control cells by 5.5 ± 0.4 ($n = 3$) times, whereas in cells treated with 100 μM sphingosine, only by 2.4 ± 0.3 times ($P < 0.02$). The CCK-OP addition released all of the stored calcium as demonstrated by the finding that the calcium ionophore, ionomycin, did not increase $[Ca^{2+}]_i$ after the CCK-OP treatment (data not shown). These results indicated that the sphingosine-induced increase in $[Ca^{2+}]_i$ resulted from release of calcium from the agonist-sensitive pool. There was then less calcium in the pool available for release by CCK-OP. The effect of sphingosine could have been either inhibition of the pool Ca^{2+} uptake mechanism or activation of the release mechanism.

For the experiments illustrated in Figs. 2 and 3 we determined the effects of sphingosine on Ca^{2+} uptake into the pool. In the experiment in Fig. 2, we first depleted the agonist-sensitive pool of calcium in acini by incubating in the absence of extracellular $CaCl_2$ and by the addition of a maximally effective concentration of carbachol. Carbachol released pool calcium as illustrated by the rapid increase in $[Ca^{2+}]_i$. Then Ca^{2+} was pumped out of the cell as illustrated by the subsequent decrease in $[Ca^{2+}]_i$ to values less than the initial. The action of carbachol was terminated with atropine. At this point we added extracellular $CaCl_2$ which was transported into the cytoplasm as indicated by the increase in $[Ca^{2+}]_i$. Because carbachol action was terminated, the Ca^{2+} in the cytoplasm was available to refill the pool [12]. Pool refilling was measured by the ability of a subsequent addition of CCK-OP to increase $[Ca^{2+}]_i$ (i.e., release Ca^{2+} from the pool into the cytoplasm) [12]. To rule out the possibility that the effect of sphingosine was due to inhibition of atropine binding, we measure the effect of sphingosine on the ability of atropine to prevent carbachol-stimulated increase in $[Ca^{2+}]_i$. Both in the presence and absence of sphingosine (100 μM) atropine prevented the ability of carbachol (100 μM) to increase $[Ca^{2+}]_i$ (not shown). When we added sphingosine (25–100 μM) to the acini before the $CaCl_2$ addition, the major effect was a dose-dependent inhibition of the CCK-OP stimulated increase in $[Ca^{2+}]_i$. In most experiments this increase was completely inhibited with 100 μM sphingosine. The effects of 25 and 50 μM sphingosine were partial. Sphingosine enhanced the increase in $[Ca^{2+}]_i$ after $CaCl_2$ addition independent of CCK-OP stimulation when 100 μM sphingosine was used. This effect was probably due to continued Ca^{2+} transport across the plasma membrane that would be expected when pool Ca^{2+} uptake is inhibited resulting in persistent depletion of pool calcium [15].

In the experiment illustrated in Fig. 3, after intracellular calcium depletion by carbachol stimulation in Ca^{2+} free media, 10 mM $CaCl_2$ was added to the extracellular media. This addition resulted in an increase in $[Ca^{2+}]_i$ to levels greater than the resting level. With the subsequent termination of carbachol action with atropine, $[Ca^{2+}]_i$ significantly decreased.

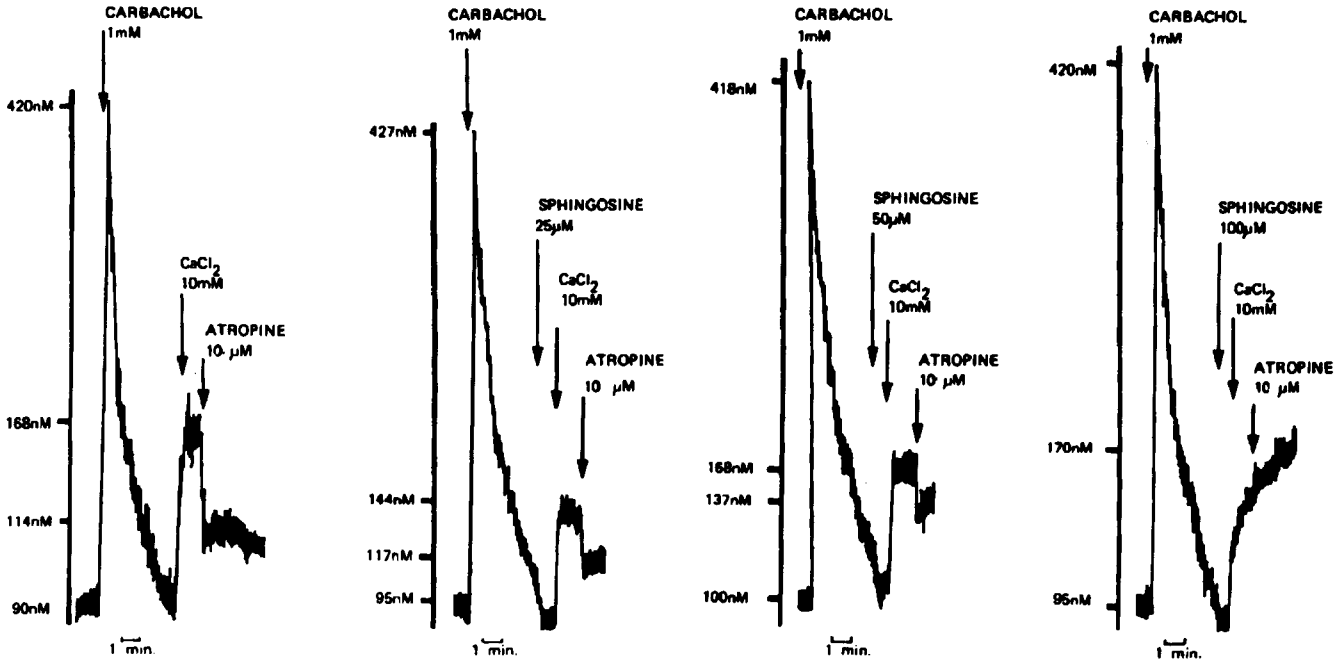

Fig. 3. Effect of sphingosine on $[Ca^{2+}]_i$ at the termination of stimulation. Fura-2/AM-loaded acini were transferred to standard incubation solution containing no added $CaCl_2$ and 0.2 mM EGTA just before measurement and incubated at 37°C. Additions were made where indicated. These are tracings of a single experiment representative of six others.

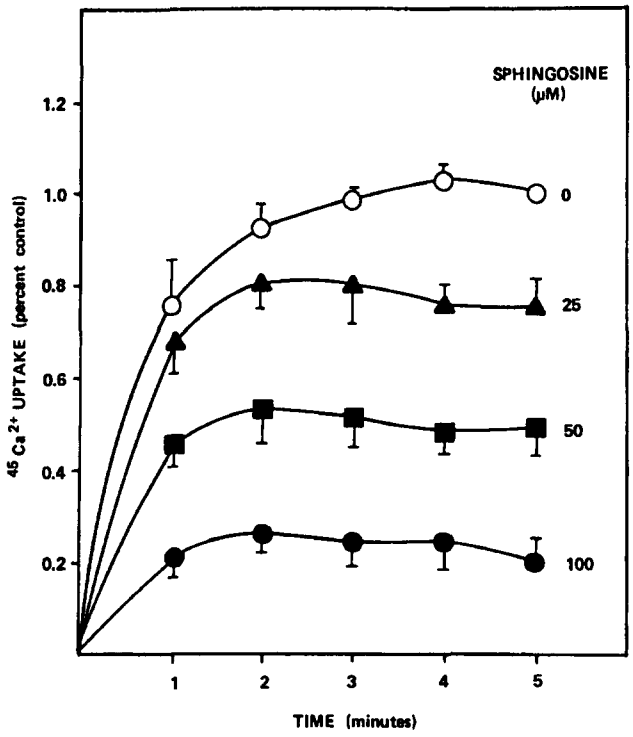

Fig. 4. Effect of sphingosine on $^{45}Ca^{2+}$ uptake in permeabilized pancreatic acini. Immediately before electrophoration, $^{45}CaCl_2$ (200 nM) and the indicated concentrations of sphingosine were added to dispersed acini. The technique of permeabilization was as described in Materials and methods. $^{45}Ca^{2+}$ uptake was measured at the times indicated. $^{45}Ca^{2+}$ accumulation in the absence of ATP was 79.0 ± 1.0% of the value for 100 μM sphingosine plus 2 mM ATP. For each experiment, values for cellular $^{45}Ca^{2+}$ were expressed as a percent of the value observed at 5 min with no added sphingosine. Results are the means of four separate experiments. Vertical bars represent 1 S.E.

This decrease could result from either a decrease in transport across the plasma membrane or increased uptake into the agonist-sensitive pool [11,12]. Our previous measurements of $^{45}Ca^{2+}$ flux into the cell demonstrated that the rate of influx is not decreased with atropine addition [11,12]. Thus, the decrease in $[Ca^{2+}]_i$ with atropine addition in Fig. 3 resulted from an increase of net uptake of Ca^{2+} into the agonist-sensitive pool. Sphingosine caused a dose-dependent inhibition of the atropine effect. 100 μM sphingosine completely inhibited the atropine effect in all experiments. These results suggested that by blocking refilling of the pool, sphingosine regulated $[Ca^{2+}]_i$.

To directly demonstrate the effect of sphingosine on Ca^{2+} uptake into intracellular stores we performed the experiment in Fig. 4. For this experiment, we first depleted the intracellular agonist-sensitive pool of Ca^{2+} by preincubating acini with carbachol in Ca^{2+}-free media. We then permeablized the plasma membrane with electroporation and measured $^{45}Ca^{2+}$ uptake in the presence or absence of sphingosine. The results demonstrated that sphingosine inhibited $^{45}Ca^{2+}$ uptake in a dose-dependent manner. 100 μM sphingosine inhibited $^{45}Ca^{2+}$ uptake by approx. 80%, in the presence of 100 μM sphingosine withdrawal of ATP decreased $^{45}Ca^{2+}$ accumulation only by 20 ± 2%.

To directly measure the effect of sphingosine on Ca^{2+}-ATPase activity we performed the experiment illustrated in Fig. 5. The results indicated that sphingosine as well as thapsigargin (a known inhibitor of microsomal Ca^{2+}-ATPase [15,23] caused inhibition of the Ca^{2+}-ATPase in our preparation of rough microsomes. The EC_{50} for the effect of sphingosine on Ca^{2+}-ATPase activity was below 25 μM (Fig. 5). This value was lower than EC_{50} for inhibition of $^{45}Ca^{2+}$ uptake (40 μM, Fig. 4). The reasons for the difference are not clear. It may be accounted for by the fact that $^{45}Ca^{2+}$ uptake was measured in permeabilized cells, while Ca^{2+}-ATPase activity in isolated microsomes. The other possible explanation is that sphingosine in-

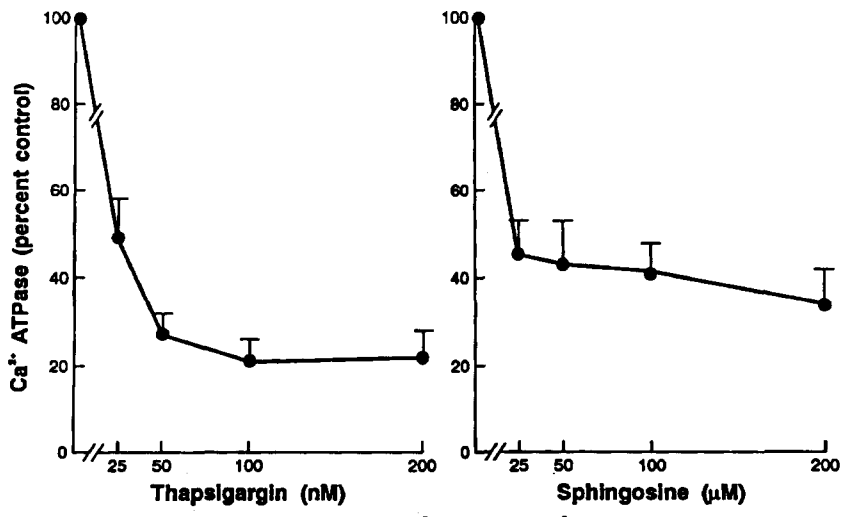

Fig. 5. Effects of thapsigargin and sphingosine on rough microsomal Ca^{2+}-ATPase. Ca^{2+}-ATPase activity was measured and values expressed as described in Materials and methods. Results are the means of four separate experiments. Vertical bars represent 1 S.E

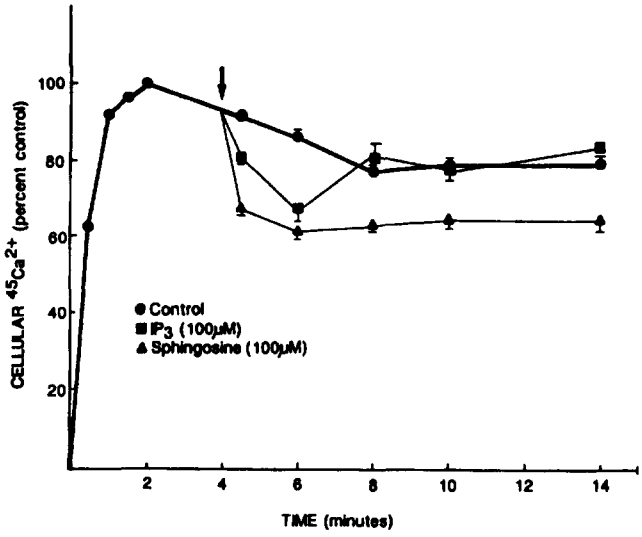

Fig. 6. Effects of sphingosine and inositol 1,4,5-trisphosphate on $^{45}Ca^{2+}$ content of permeablized pancreatic acini. Dispersed acini were permeabilized and loaded with 200 nM $^{45}CaCl_2$ as described in Materials and methods. Sphingosine, inositol 1,4,5-trisphosphate (IP_3) or the combination of sphingosine and IP_3 were added at 4 min of incubation. Cellular $^{45}Ca^{2+}$ was measured at the times indicated. For each experiment, values for cellular $^{45}Ca^{2+}$ were expressed as a percent of the value observed at 2 min. Results are the means of four separate experiments. Vertical bars represent 1 S.E.

hibits not only Ca^{2+} uptake but Ca^{2+} release from intracellular compartments as well. In rabbit skeletal muscle fibers sphingosine was found to inhibit sarcoplasmic reticulum Ca^{2+} release by a direct effect on ryanodine receptor [26]. We haven't found this effect in pancreatic acinar cells; sphingosine (5–100 μM) did not produce any effect on caffeine (1 mM)-induced $[Ca^{2+}]_i$ rise (not shown).

In order to determine if the effect of sphingosine on intracellular pool Ca^{2+} was due only to inhibition of uptake, we performed the experiment illustrated in Fig. 6. For this experiment, permeabilized acini were loaded to equilibrium with 200 μM $^{45}CaCl_2$. Then the effects of sphingosine, inositol 1,4,5-trisphosphate (IP_3) and the combination of sphingosine and IP_3 on cellular $^{45}Ca^{2+}$ content were measured. Sphingosine (100 μM) alone did not cause a rapid decrease in $^{45}Ca^{2+}$, which is an agreement with the data obtained earlier [27]. The combination of the of IP_3 and sphingosine caused a larger decrease in stores $^{45}Ca^{2+}$ than observed with IP_3 alone and the response was converted from a transient one to a sustained one. These results indicated that sphingosine specifically prevented uptake of Ca^{2+} into the stores and did not directly cause Ca^{2+} release.

In conclusion, the results from the present series of experiments indicated that sphingosine had a specific effect to inhibit Ca^{2+} reloading of the agonist-sensitive pool by inhibiting microsomal Ca^{2+}-ATPase. Interestingly, this is an effect shared by the tumor promoter, thapsigargin [15]. Thus, sphingosine may represent an endogenous thapsigargin. Our experiments demonstrated that this effect of sphingosine can maintain the intracellular stores in a Ca^{2+} depleted state after the transient Ca^{2+} release caused by IP_3. This effect, in turn, increases $[Ca^{2+}]_i$ and Ca^{2+} influx [11–15]. Our results raise the possibility that endogenous sphingosine or a sphingosine-like agent mediates some of the actions of the agonist on cellular Ca^{2+} transports. Such a suggestion would be in agreement with recent reports that sphingosine and sphingosine metabolites cause $[Ca^{2+}]_i$ oscillations in pancreatic acini [27].

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