ACTA NEUROBIOLOGIAE EXPERIMENTALIS

Acta Neurobiol. Exp. 1996, 56: 507-513

Sphingosine stimulates calcium mobilization and modulates calcium signals evoked by thapsigargin in glioma C6 cells

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Abstract. The effect of sphingosine on intracellular calcium signalling in glioma C6 cells was studied with Fura-2 video imaging technique. Sphingosine had a direct effect on changes in cytosolic Ca^{2+} concentration only when applied at a high concentration of 100 μM, causing the cytosolic Ca^{2+} level to rise. However, at a much lower concentration of 15 μM sphingosine diminished calcium responses triggered by thapsigargin (a specific inhibitor of calcium pump in the endoplasmic reticulum) and ionomycin (calcium ionophore). Since responses to thapsigargin and ionomycin were blocked in Ca^{2+}-free medium, we postulate that sphingosine is acting on the intracellular calcium stores. Additionally, sphingosine (at 15 μM and 100 μM) markedly decreases thapsigargin-induced sustained elevation in cytosolic Ca^{2+} concentration, indicating its inhibitory effect on thapsigargin-evoked Ca^{2+} influx. Sphingosine is a known inhibitor of protein kinase C and the involvement of this enzyme is postulated in the modulatory effects of sphingosine on intracellular calcium dynamics.

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Key words: sphingosine, calcium signals, thapsigargin, glioma C6 cells

INTRODUCTION

Sphingosine is a natural constituent of cells and attracts considerable experimental attention. As a naturally occurring sphingoid breakdown product of cellular sphingolipids, sphingosine has recently emerged as a bioregulatory molecule. The main interest in possible function of sphingosine stems from the discovery that sphingosine is a potent inhibitor of protein kinase C (PKC) (Hannun et al. 1986) and interferes with biological action of PKC in multiple cell systems (Merrill 1991). It has been suggested, therefore, that sphingosine may play a role as an endogenous modulator of cell function (for review, see Hannun and Linard 1993, Divecha and Irvine 1995). Sphingosine also has complex biological effects which seem to be PKC-independent. Among many biological activities of sphingosine there are mitogenic effects, stimulation of proliferation, and intracellular Ca^{2+} mobilization by the release of Ca^{2+} from internal stores, although the mechanism of the last process remains unclear (Divecha and Irvine 1995).

In nonexcitable cells the increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is due to a release of Ca^{2+} from intracellular stores and Ca^{2+} influx. The depletion of intracellular stores occurs by the action of inositol 1,4,5-trisphosphate ($InsP_3$), the second messenger generated by phospholipase C-linked agonists (Berridge 1993). Intracellular Ca^{2+} mobilization occurs also with the application of such drugs as thapsigargin.

Thapsigargin, a plant sesquiterpene lacton, is a potent and specific inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase, pumping Ca^{2+} into this structure (Thastrup et al. 1990, Sabała et al. 1993). Thapsigargin blocks this enzyme and Ca^{2+} leaks from the lumen of the endoplasmic reticulum that cannot be refilled again. Thus, thapsigargin should result in depletion of intracellular stores and in the increase of Ca^{2+} level in the cytosol, without generation of inositol 1,4,5-trisphosphate ($InsP_3$). This transient phase of the increase in cytosolic free Ca^{2+} concentration is followed in many cells by the influx of Ca^{2+} from extracellular space (sustained phase).

In our previous investigation we demonstrated the modulatory effect of phorbol ester (12-O-tetradecanoylphorbol-13-acetate, TPA), a PKC activator, on thapsigargin-induced changes in cytosolic Ca^{2+} concentration in glioma C6 cells (Barańska et al. 1995). Glioma C6 cells belong to the type of nonexcitable cells (Barańska et al. 1995). The aim of the present study was to examine whether sphingosine, an inhibitor of PKC, could release Ca^{2+} from intracellular (endoplasmic reticulum) stores and modulate Ca^{2+} signals evoked by thapsigargin in these cells. A part of these results has been previously presented in abstract form (Chaban et al. 1995).

METHODS

Chemicals

Minimum Essential Medium (MEM), calf serum and phosphate buffered saline (PBS) were from Gibco BRL, UK. Bovine serum albumin (BSA), EGTA, ionomycin, D-(+)-erythro-trans-sphingosine (the naturally occurring isomer) and cell dissociation solution were purchased from Sigma Chemical Co., St.Louis, USA. Thapsigargin was from LC Services Corporation, Woburn, USA and Fura-2 AM was obtained from Molecular Probes, Inc.

Cell culture

Glioma C6 cells were cultured in MEM supplemented with 10% (v/v) calf serum, penicillin (50 IU/ml), streptomycin (50 μg/ml) and 2 mM L-glutamine under humidified atmosphere of 5% CO_2 at 37°C. The cells were passaged when confluent by using nonenzymatic cell dissociation solution, and the medium was changed twice a week. Cells for experiments were placed on glass coverslips and cultured for three days under the same conditions.

Measurement of intracellular calcium

Intracellular Ca^{2+} level was measured as described previously (Barańska et al. 1995) with minor modifications. Cells on coverslips were

washed once with PBS and once with the following mixture (buffer A): 137 mM NaCl, 2.7 mM KCl, 1 mM Na_2HPO_3, 25 mM glucose, 20 mM Hepes (pH 7.4), 1 mM $MgCl_2$, 1% (v/v) bovine serum albumin and 2 mM $CaCl_2$. Only in the experiments concerning the absence of external Ca^{2+} 500 μM EGTA was added instead of 2 mM $CaCl_2$ (as indicated in legends to figures). The cells were then incubated at 37°C in buffer A for 30 min with 1 μM Fura-2 AM. Thereafter the cells were washed three times with buffer A. Coverslips were mounted in a chamber over a Nikon Diaphot inverted-stage microscope equipped with a x 40 oil-immersion fluorescence objective lens. All substances were added as solutions in buffer A (all concentrations indicated in figures). Digital fluorescent microscopy was used to determine the spatial distribution of $[Ca^{2+}]_i$. Experiments were carried out on video imaging system (MagiCal, Applied Imaging Ltd.) in the Laboratory of Electron Microscopy, Nencki Institute of Experimental Biology. The cells were alternatively illuminated with 340 and 380 nm wavelengths of light from a xenon lamp. The emitted light was passed through a 510 nm barrier filter into an image-intensified camera (Extended ISIS, Photonic Science). The 340 nm and 380 nm images (256 grey levels) were software averaged and captured every 2.85 s. The 340 nm and 380 nm signals were examined for real changes in $[Ca^{2+}]_i$. Ratio (R) values were converted to an estimate of $[Ca^{2+}]_i$ using the formula (Grynkiewicz et al. 1985):

$$[Ca^{2+}]_i = K_d \cdot \beta \cdot (R - R_{min}) / (R_{max} - R)$$

Intracellular calibration was carried out by addition of 4 μM ionomycin to glioma C6 cells placed in solutions containing 2 mM Ca^{2+} ($R_{max} = 2.3$) and in the absence of calcium with 5 mM EGTA ($R_{min} = 0.3$). The β value was 4.5 and K_d of 224 nM was assumed. Data processing and ratio values conversion to an $[Ca^{2+}]_i$ were carried out using Tardis V8.0.

Each trace shown in figures represents a mean value from one representative experiment, and each experiment was conducted on at least four separate occasions.

RESULTS

Figure 1 presents the effect of sphingosine on the changes in $[Ca^{2+}]_i$ level in Fura-2 AM loaded glioma C6 cells and on thapsigargin-induced Ca^{2+} mobilization in these cells. Treatment with 15 μM sphingosine did not produce a visible increase in $[Ca^{2+}]_i$, but caused a decease of the thapsigargin-induced Ca^{2+} signal (Fig. 1A). Thapsigargin in the presence of extracellular Ca^{2+} produced a rapid increase in $[Ca^{2+}]_i$, which consisted of two phases: a transient, initial phase associated with the depletion of intracellular stores, and a sustained phase manifested in the sustained elevation in $[Ca^{2+}]_i$ as a result of the Ca^{2+} influx to the cell (Fig. 1C). In the absence of external Ca^{2+}, thapsigargin caused only a transient rise in $[Ca^{2+}]_i$ (transient phase) (Fig. 1F).

As is shown in Fig. 1A, sphingosine added to the cells before thapsigargin diminished thapsigargin-induced cytosolic Ca^{2+} mobilization by the reduction of the second, sustained phase of $[Ca^{2+}]_i$. The response of the cells to 100 μM sphingosine was similar, although the cells manifested a slow increase in $[Ca^{2+}]_i$ (up to 200 nM), which then decreased over 4-6 min (Fig. 1B). The addition of thapsigargin to such cells elicited a very small rise in $[Ca^{2+}]_i$, significantly smaller than that observed for thapsigargin alone (Fig. 1C) and again consisted of a transient phase only. All of these effects were manifested in the presence of external Ca^{2+}. In Ca^{2+} free medium, both 15 μM and 100 μM sphingosine added prior to thapsigargin caused a decrease in thapsigargin-induced $[Ca^{2+}]_i$ (Fig. 1D and E).

Thus, sphingosine diminished the cytosolic Ca^{2+} concentration in the cells treated with thapsigargin not only in the presence but also in the absence of extracellular Ca^{2+} (Fig. 1). These data suggested that sphingosine might affect not only thapsigargin-induced influx of Ca^{2+}, but also the release of Ca^{2+} from intracellular stores. To check whether sphingosine could deplete these stores prior to addition of thapsigargin (then sphingosine-mediated release of Ca^{2+} would occur so slowly that only a small elevation in cytosolic Ca^{2+} level could be observed), in the next experiments the cells were tested in the ab-

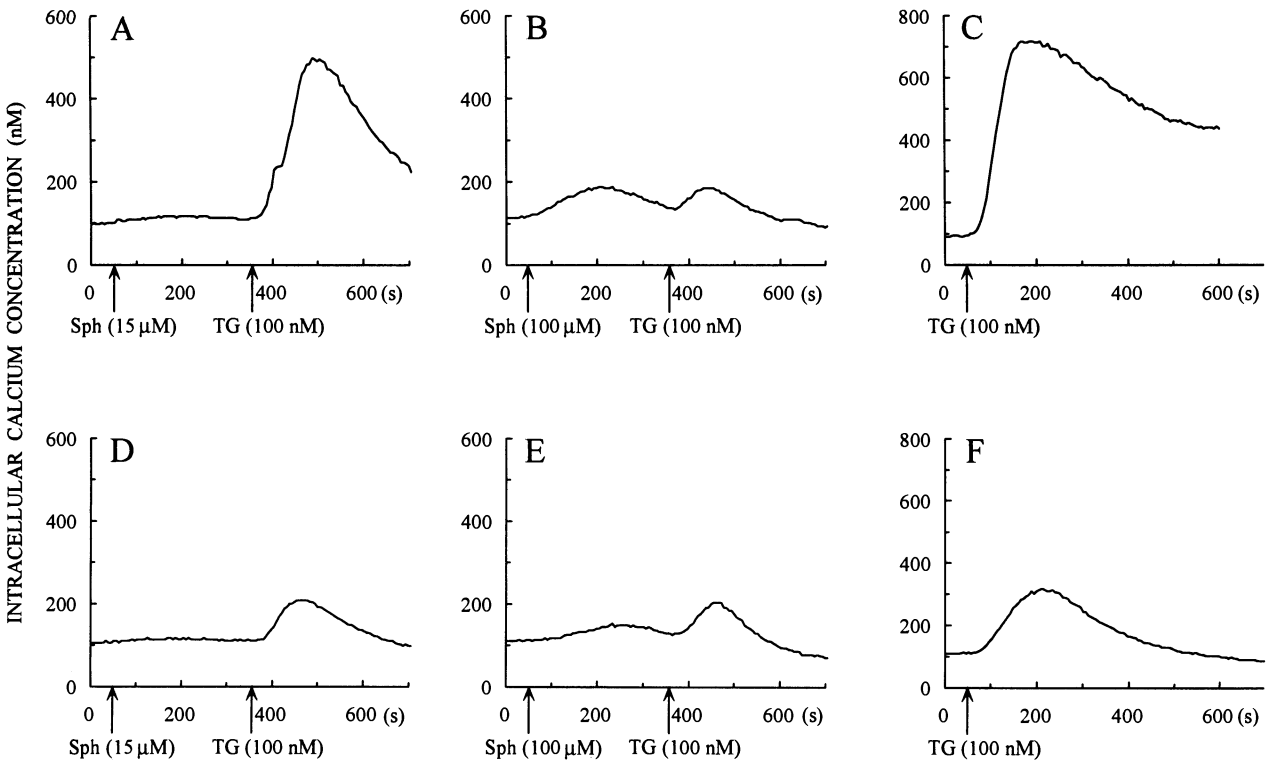

Fig. 1. Effect of sphingosine (15 μM and 100 μM) on changes in intracellular Ca^{2+} concentration and on thapsigargin-induced Ca^{2+} mobilization in glioma C6 cells. A, B, C, buffer A containing 2 mM $CaCl_2$; D, E, F, buffer A without $CaCl_2$ and with 500 μM EGTA. Each trace represents mean value for one representative experiment for: A, 15 cells; B, 13 cells; D, 13 cells; E, 16 cells; F, 15 cells. Each experiment was conducted on five separate occasions. Sphingosine (Sph) and thapsigargin (TG) were added as indicated by arrows.

sence of external Ca^{2+} and with thapsigargin or sphingosine added prior to ionomycin, the Ca^{2+} ionophore. It was found that in control, untreated cells, ionomycin released Ca^{2+} from intracellular stores (Fig. 2A). On the other hand, when thapsigargin was added prior to ionomycin and emptied Ca^{2+} stores, then ionomycin was unable to produce any additional rise in cytosolic Ca^{2+} concentration (Fig. 2B). The addition of sphingosine (15 and 100 μM) caused a small increase in $[Ca^{2+}]_i$, and ionomycin added 5 min after sphingosine was able to release these ions (Fig. 2C and D). However, in the case of cells treated previously with 15 μM sphingosine (Fig. 2C), or, in particular, with 100 μM sphingosine (Fig. 2D), the release of Ca^{2+} by ionomycin added after sphingosine was distinctly smaller than that produced by ionophore alone in control cells (Fig. 2A). These data indicate that sphingosine can indeed release Ca^{2+} from intracellular stores and that this release is partial.

It should be added that all of the cells exposed to thapsigargin and ionomycin responded homogeneously in the $[Ca^{2+}]_i$. Whenever sphingosine was used Ca^{2+} responses of the individual cells showed a certain heterogeneity, but most of the cells (70%) responded in the manner shown in Figs. 1 and 2.

DISCUSSION

Sphingosine has been proposed as a modulator of Ca^{2+} signals in cells, mediating Ca^{2+} release from the intracellular pool sensitive to $InsP_3$. This release has been observed in permeabilized cells of the DDT1MF2 smooth muscle (Ghosh et al. 1990, 1994), intact parotid acinar cells (Sugiya and Furuyama 1991), Swiss 3T3 fibroblasts (Zhang et al. 1991, Olivera et al. 1994), pancreatic accinar cells (Yule et al. 1993), human fibroblasts (Chao et al. 1994) and thyroid FRTL-5 cells (Tornquist and Ekokoski

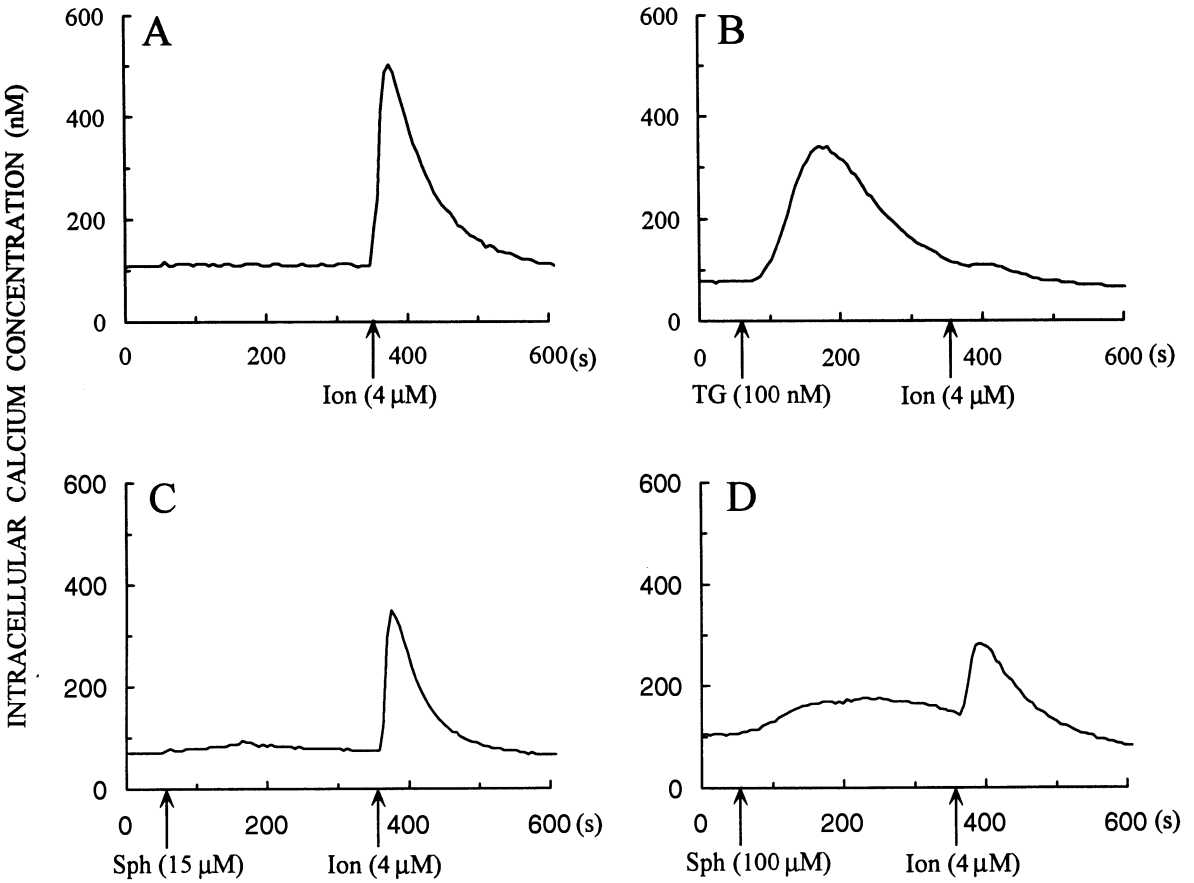

Fig. 2. Effect of sphingosine (Sph) and thapsigargin (TG) on Ca^{2+} signals elicited in glioma C6 cells by 4 μM ionomycin (Ion) in the absence of extracellular Ca^{2+}. A, control cells treated with ionomycin; B, cells treated with 100 nM thapsigargin prior to ionomycin; C, cells treated with 15 μM sphingosine prior to ionomycin; D, cells treated with 100 μM sphingosine prior to ionomycin. Each trace represents mean value for one representative experiment for: A, 19 cells; B, 14 cells; C, 20 cells; D, 15 cells. Each experiment was conducted on at least four separate occasions.

1994). Recently, Olivera et al. (1994) have found that this effect is produced only by naturally occurring isomers of sphingosine (D-(+)-erythro-isomers) and does not appear to be PKC- dependent.

However, mechanisms of sphingosine action remain poorly understood and are still a subject of investigation. It has been suggested that in cultured smooth muscle cells, sphingosine is converted in the endoplasmic reticulum membrane to sphingosine-1-phosphate and that this metabolite affects calcium release (Ghosh et al. 1990, 1994). This phosphorylated metabolite of sphingosine has been also proposed as a putative intracellular second messenger involved in the regulation of the level of sphingosine in the cell and in control of Ca^{2+} mobilization by the release of Ca^{2+} from intracellular stores in Swiss 3T3 fibroblasts (Olivera et al. 1994). Instead, in rat pancreatic accinar cells, sphingosylphosphorylcholine has been postulated as the sphingosine metabolite responsible for Ca^{2+} oscillations (Yule at al. 1993). On the other hand, Chao et al. (1994) have proposed that in human fibroblasts, besides sphingosine metabolites, sphingosine itself induces Ca^{2+} release. The authors have suggested that sphingosine generates $InsP_3$ that interacts with $InsP_3$ receptors in the endoplasmic reticulum membranes and releases Ca^{2+} to the cytosol.

Nevertheless, the present study indicated that sphingosine modified the cytosolic Ca^{2+} concentration and could release Ca^{2+} from intracellular stores in glioma C6 cells. This effect was obtained at high (100 μM) and low (15 μM) concentrations of sphin-

gosine. However, sphingosine-induced Ca^{2+} release was low and occurred so slowly that it did not produce typical transient and sustained elevation in the cytosolic Ca^{2+} concentration, as thapsigargin did. It could be revealed due to action of ionomycin, the Ca^{2+} ionophore, added to the cells in the absence of extracellular Ca^{2+} after previous treatment with sphingosine. However, even a low sphingosine concentration (15 μM) was high enough to diminish to some extent thapsigargin-mediated Ca^{2+} release, whereas 100 μM sphingosine strongly modified and decreased Ca^{2+} mobilization from intracellular stores induced by thapsigargin.

Furthermore, the data described here showed that in glioma C6 cells sphingosine affected not only thapsigargin-induced internal Ca^{2+} release, but also markedly slowed down and lowered thapsigargin-induced Ca^{2+} entry. Similarly, sphingosine specifically inhibited the Ca^{2+} influx generated by thapsigargin in Jurkat T cell line (Breittmayer et al. 1994). Since, in these cells, sphingosine alone did not modify the cytosolic Ca^{2+} concentration, the authors proposed that this drug decreased thapsigargin-induced Ca^{2+} influx by the activation of the Ca^{2+} extrusion. On the other hand, most recently Balasubramanyam and Gardner (1995) reported that in Jurkat T lymphocytes an activation of PKC by phorbol ester activated the Ca^{2+} extrusion process by the stimulation of the plasma membrane Ca^{2+} pump.

We previously showed that in glioma C6 cells both PKC activation by TPA and PKC down-regulation markedly decreased the Ca^{2+} entry generated by thapsigargin (Barańska et al. 1995). These previous observations and results of the present study allowed us to speculate that in glioma C6 cells the inhibition of Ca^{2+} flows by sphingosine may be PKC-dependent. Glioma C6 cells belong to nonexcitable cells (Barańska et al. 1995). One can speculate that, similar to what has been reported for excitable cells (Rasmusen et al. 1992), in glioma C6 cell line PKC also affects the Ca^{2+} influx and Ca^{2+} efflux, leading to an increase of Ca^{2+} cycling across the plasma membrane and maintaining Ca^{2+} homeostasis. Thus, when PKC is activated by TPA, thapsigargin-induced entry of Ca^{2+} would be balanced by a similar rate of efflux and the second, sustained phase of Ca^{2+} mobilization could not be seen (Barańska et al. 1995). On the other hand, when PKC activity is inhibited by PKC down-regulation (Barańska et al. 1995), or by sphingosine treatment (the present data), both the influx and efflux of Ca^{2+} should be diminished and again the sustained phase of thapsigargin-generated Ca^{2+} mobilization (the Ca^{2+} influx) could not be observed. In conclusion, we suggest that in glioma C6 cells sphingosine may modulate Ca^{2+} signals in a PKC-dependent manner, although the mechanism of the Ca^{2+} release from the intracellular stores is most probably PKC-independent. Further study should elucidate and answer these still open questions.

In addition, it is worth noting that the C6 glioma cell line can be used as a very convenient, *in vitro* model for the study of glial cell properties (Coyle 1995). Therefore, the close examination and better understanding of calcium homeostasis in glioma C6 cells would be helpful in gaining insight to the physiological meaning of calcium signalling in glia.

ACKNOWLEDGEMENTS

This study was supported by a grant from the State Committee for Scientific Research for the Nencki Institute and grant No 4 P05A 04408 KBN from the State Committee for Scientific Research in Poland. P.S. is the recipient of the 1996' Fundation for Polish Science fellowship programme.

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Received 12 February 1996, accepted 22 February 1996

This paper is dedicated to Professor Stella Niemierko on the occasion of her 90th birthday, with esteem and admiration