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Sphingolipids Increase Calcium Concentration in Isolated Rat Liver Nuclei

R. E. Catalán,*[,1] B. G. Miguel,† M. C. Calcerrada,† S. Ruiz,† and A. M. Martínez†

**Departamento de Biología Molecular, Centro de Biología Molecular "Severo Ochoa" (CSIC-UAM), Universidad Autónoma de Madrid, E-28049, Spain; and †Departamento de Bioquímica y Biología Molecular I, Facultad de Químicas, Universidad Complutense de Madrid, E-28040 Madrid, Spain*

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The sphingolipids, sphingosine (SPH), sphingosylphosphorylcholine (SPC) and psycosine induce a rapid and transient rise in nuclear free Ca^{2+} concentration in a dose dependent manner. To determine whether these sphingolipids act by a IP_3-dependent pathway, we tested the increase of Ca^{2+} in the presence of heparin, an antagonist of IP_3 receptor or U70122, an inhibitor of phospholipase C. Results indicate that the effect of both SPH and SPC, but not that of psychosine, is partially mediated by IP_3 production. The sphingolipid-induced Ca^{2+} mobilization was unaffected by the inhibition of protein kinase C, but was totally abolished in the presence of nimodipine, a L-type Ca^{2+} channel inhibitor. The results could indicate the existence of a sphingosine-gated Ca^{2+}-permeable channel in liver nuclei.

Sphingolipid metabolites, including sphingosine (SPH) and ceramide have recently been proposed a second messengers in signal transduction pathways (1). The generation of intracellular ceramide by the action of sphingomyelinase, may modulate several processes including cell division, apoptosis, secretion of prostaglandin E_2 and activation of protein kinases and phosphoprotein phosphatases (1). Deacylation of ceramide produces sphingosine (SPH) which inhibits protein kinase C (2) and induces intracellular calcium mobilization (3). Although the mechanism remains unsufficiently elucidated, some authors have suggested that SPH generates IP_3 that interacts with IP_3 receptors in the endoplasmic reticulum membranes and releases Ca^{2+} to the cytosol (4). In addition, some lysosphingolipids such as sphingosylphosphorylcholine (SPC) and galactosylsphingosine (psychosine) induced Ca^{2+} mobilization from IP_3 sensitive pool in different cell types (5-7). It has been suggested, therefore, that sphingolipids may play a role of endogenous modulators of cell function (1,2). When added exogenously to cell SPH is converted to sphingosine-1-phosphate (SPP) that is likely to mediate some, but not all, of the effects of SPH (8).

The regulation of intracellular Ca^{2+} has been extensively studied (9) and the relationship between cytosolic Ca^{2+} changes and nuclear Ca^{2+} levels has drawn considerable attention (9,10). It is known that Ca^{2+} plays a crucial and complex role in nucleus and that it has been implicated in diverse nuclear functions including gene transcription, DNA synthesis and nuclear envelope breakdown (11). Although the nuclear membrane is considered to be very permeable to molecules larger than 20 kDa (12), and therefore simple ion like calcium diffuses freely in and out of the nucleus, differences between Ca^{2+} concentration in the nucleoplasm and the cytosol have been observed (13). Some data indicated that, in general, cytosolic and nucleoplasmic Ca^{2+} concentrations equilibrate rapidly (14) but other authors have proposed that the nucleus is largely insulated from cytosolic Ca^{2+} changes (15).

The presence and function of phospholipids in the nucleus have been reported (16). Data involving SP concentration in DNA stabilization have been published (17). More recently, Neitchere et al. (18) have reported the presence of sphingomyelinase activity in rat liver nuclei.

In the present paper, our study was focused on the Ca^{2+} mobilizing action of SPH and other related sphingolipids in rat liver nuclei.

MATERIALS AND METHODS

Materials. Fura-2-dextran was purchased from Molecular Probes. Sphingolipids, heparin and IP_3 were obtained from Sigma. Nimodipine and U 70122 were from Calbiochem.

Preparation of rat liver nuclei. Rat liver nuclei were isolated from starved male Wistar rats, basically as previously described (19). Livers were homogenized in solution A (50 mM triethanolamin-HCl, pH

[1] To whom correspondence should be addressed. Fax: (341) 3974870.

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7.5, 25 mM KCl, 5 mM $MgCl_2$, 0.5 mM PMSF and 1 mM DTT) and 0.25 M sucrose. The homogenate was filtered through four cheesecloth, and 1 M DTT was added. The nuclei were pelleted by centrifugation at 800 × g for 15 min. The resulting pellet was resuspended in the same solution and two volumes of solution A supplemented with 2.3 M sucrose were added. The mixture was layered on top of 5 ml of solution A supplemented with 2.3 M sucrose. The interface was disrupted with a Pasteur pipette and the samples were centrifuged at 124000 × g for 1h at 4°C. The resulting pellets were resuspended in a solution containing 50 mM Hepes, pH 7.0, 125 mM KCl, 2 mM K_2HPO_4, 4 mM $MgCl_2$ and 0.1 mM EGTA.

The purity of the nuclei thus obtained was controlled by microscopic techniques and by marker enzymes analysis to assess the level of contamination by plasma membranes and microsomes.

Measurement of calcium. Isolated nuclei were loaded with 20 μM Fura-2-dextran for 10 min at 4°C in the presence of 1 mM ATP as previously described (20). The loaded nuclei were washed by centrifugation at 800 × g for 10 min, and resuspended in a medium containing 0.25 mM sucrose, 2 mM EGTA, 2 mM EDTA, 4 mM K_2HPO_4, 4 mM $MgCl_2$, 50 mM Tris HCl and 2.08 mM $CaCl_2$ to achieve 1 μM free calcium concentration. Samples were placed in a 1 cmm^2 quartz cuvette and experiments were performed at room temperature. Changes in Ca^{2+} concentration were monitored by a fluorometer, the ratio of excitation wavelengths 350/380 nm with emission cutoff at 510 nm. The calcium concentration was calculated according to Grynkiewicz et al. (21).

RESULTS AND DISCUSSION

The effects of sphingosine derivatives on the changes in Ca^{2+} concentration in loaded Fura-2-dextran nuclei was studied. Treatment with 30 μM SPH, SPC or psychosine, rapidly increased Ca^{2+} concentration in nucleoplasm (Fig. 1). This effect was transient decaying to a steady Ca^{2+} level which remained elevated over baseline. We must point out that this effect resulted to be ATP-dependent, since it was reduced in the absence of 1 mM ATP. In this condition, the slight increase observed rapidly returned to basal levels. This observation is consistent with the endoplasmic-reticulum type Ca^{2+} pump ATP described in liver nuclei (22).

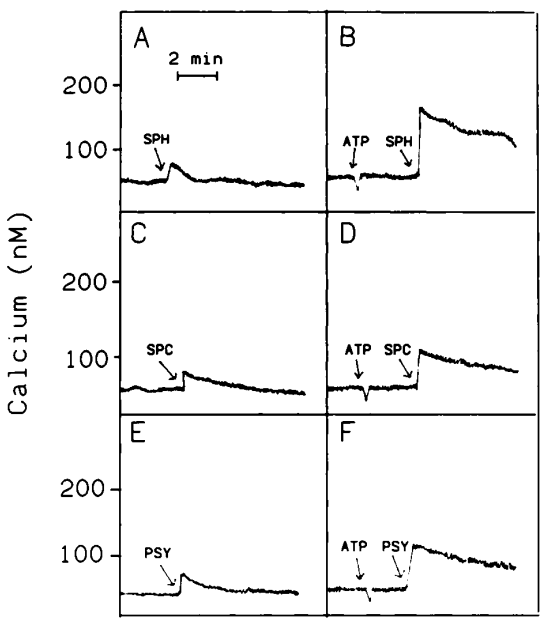

FIG. 1. Effect of sphingolipids on Ca^{2+} concentration in liver nuclei. Isolated nuclei labeled with Fura-2-dextran were treated with 30 μM sphingolipids in the absence (A) or presence (B) of 1 mM ATP. Ca^{2+} concentration was measured as described in Materials and Methods. ATP or sphingolipids were added as indicated by the arrows. Each trace is a representative from at least three experiments.

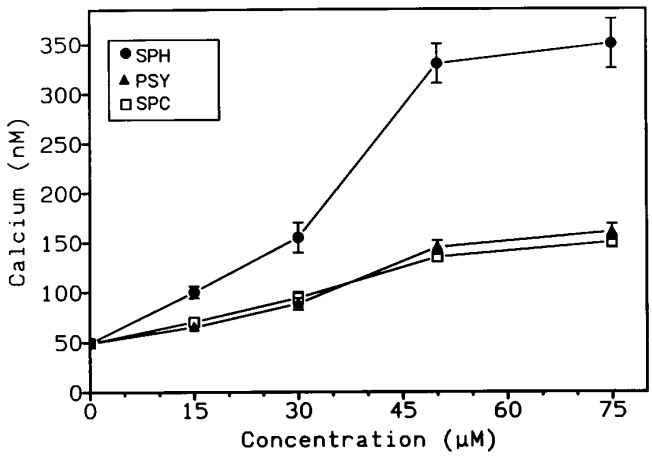

FIG. 2. Dose–response for sphingolipid-induced Ca^{2+} changes in nucleoplasm. Isolated nuclei were treated with different concentrations of SPH, SPC or psychosine. Changes in Ca^{2+} concentration were measured as described in Materials and Methods. Data represent the mean ± SE of three determinations.

The dose-response relationship for the peak response of Ca^{2+} concentration to SPH, SPC and psychosine was analyzed (Fig. 2). Nucleoplasmic Ca^{2+} concentration increased at 15 μM agonist and reached the maximum response at 50 μM lipid concentration. The SPH was the most effective lipid assayed. The highest concentrations (75 μM) evoked an increase in Ca^{2+} concentration from 50 nM to 350 nM. The sphingolipid concentrations used in these experiments were similar to those described as effective in several cell types (23).

It is possible that sphingolipids mobilize Ca^{2+} through a modulatory action on the IP_3 pathway (4), or that they could act in a completely independent manner, as recently suggested for SPP in fibroblasts (24). Taking into account that nucleus contains IP_3 receptors and the necessary machinery for IP_3 production (11), we investigated the role of IP_3 on SPH derivatives-stimulated Ca^{2+} concentration. Treatment with 100 μg/ml heparin, reported as an antagonist of IP_3 receptor (25), 2 min prior to the addition of sphingolipids (30 μM), partially inhibited the effects of SPH and SPC, but not those of psychosine (Fig. 3). Additionally, the treatment with 10 μM U70122, a potent phospholipase C inhibitor (26), also partially inhibited the effects of both SPH and SPC but did not have any effect in the case of psychosine (Fig. 3). When the nuclei were pretreated

with IP_3, the Ca^{2+} response to SPH was partially reduced (data not shown). Taken together, these findings indicate that SPH- and SPC-induced Ca^{2+} mobilization in nucleoplasm is carried out by at least two different mechanisms, one of them involving the generation of IP_3 by phospholipase C activation. The psychosine-induced Ca^{2+} mobilization appears to be completely IP_3 independent. Psychosine is a sphingosine derivative which is galactosylated at the 1-carbon, thus preventing phosphorylation at that site. The present data suggest that SPH and SPC may act through their conversion in SPP as described in some cells (6). To test this possibility, experiments in the presence of DL-*threo*-dihydrosphingosine, an inhibitor of sphingosine kinase (27) were performed. The lack of effect of the inhibitor indicated that the generation of SPP is not involved in the SPH action (Table 1). Although sphingosine kinase activity has not been detected in nuclei, it is possible that SPP can be produced by the enzyme, since it is located at the level of the endoplasmic reticulum (7) and it is generally accepted that the outer nuclear membrane has endoplasmic reticulum properties and is continuous with the endoplasmic reticulum membranes (11).

TABLE 1

Effect on Sphingosine on Ca^{2+} Concentration in Nuclei

Treatment	Ca^{2+} (nM)
None	48 ± 7
SPH	150 ± 10
DL-*threo*-DHS	52 ± 7
DL-*threo*-DHS + SPH	146 ± 9

Isolated nuclei labeled with Fura-2-dextran were treated with 30 μM DL-*threo*-dihydrosphingosine (DL-*threo*-DHS) 2 min prior to the treatment with 30 μM SPH. Data represent the mean ± SE of three determinations.

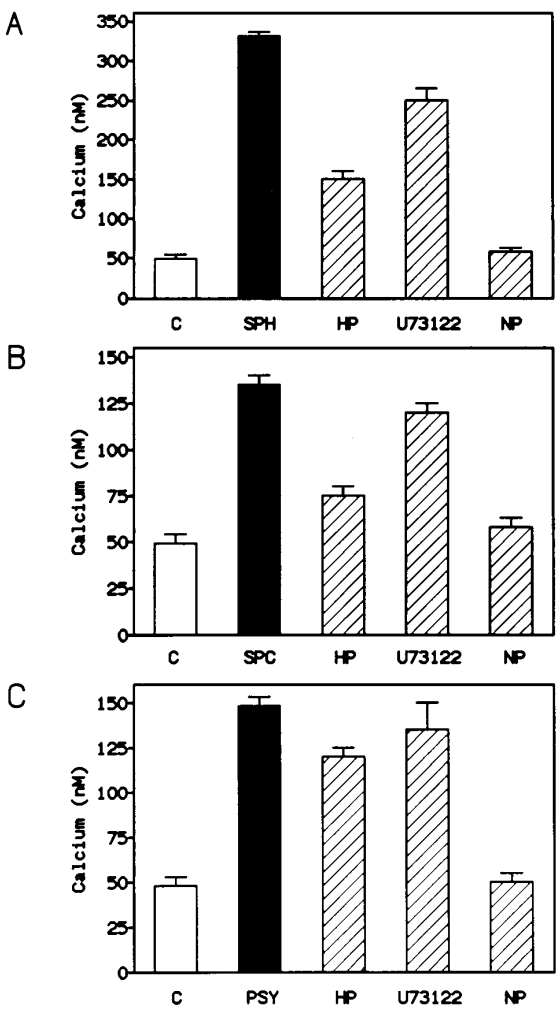

FIG. 3. Effects of several inhibitors on sphingolipid-induced Ca^{2+} mobilization in nucleoplasm. Isolated nuclei were treated with 10 μM U73122 or 100 μg/ml heparin or 10 μM nimodipine prior to the addition of SPH (A), SPC (B) or psychosine (C). Changes in Ca^{2+} concentration were measured as described in Materials and Methods. Data represent the mean ± SE of three determinations.

We have also investigated the action of nimodipine, a L-type Ca^{2+} channel inhibitor, on sphingolipid-stimulated nuclear Ca^{2+} concentration. Results in Fig. 3 indicate that 10 μM nimodipine abolished the effects of 30 μM sphingolipids. This fact may suggest the existence of a Ca^{2+} channel dihydropyridine-sensitive in liver nuclei. In this sense, a novel intracellular sphingolipid-gated calcium-permeable channel with unique pharmacological properties has been chracterized (28).

The present finding that sphingosine derivatives modulate nuclear Ca^{2+} suggest a potential role of this class of lipids, considered as second messengers, in the nuclear function, and might indicate the existence of a novel Ca^{2+} channel sphingolipid-sensitive in nuclear membrane.

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