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Sphingolipid derivatives modulate intracellular Ca^{2+} in rat synaptosomes

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Abstract. Sphingosylphosphorylcholine (SPC) induces a rapid increase of intracellular Ca^{2+} concentration in isolated synaptosomes. This effect is dose-dependent and is also dependent on extracellular Ca^{2+}. Sphingosine (SPH) has a smaller effect and treatment with psychosine (PSY) is ineffective, which suggests that phosphorylation of the 1-carbon of SPH is required for the SPC to act as a Ca^{2+} release agonist in synaptosomes. Experiments performed in the presence of heparin or ryanodine indicate that SPC-elicited Ca^{2+} release is not mediated by IP_3 or ryanodine receptors. Finally, our results show that the effect of SPC on Ca^{2+} concentration is nimodipine-sensitive, suggesting that SPC possibly activates a specific sphingolipid-gated Ca^{2+} channel in synaptosomes.

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Sphingolipids are emerging as an important new class of lipid second messenger (Spiegel and Milstein 1995). The generation of intracellular ceramide from sphingomyelin by the action of sphingomyelinase may modulate several processes, such as cell growth and cell differentiation (Kolesnick and Golde 1994). Further metabolites of ceramide, such as sphingosine (SPH) and sphingosine-1-phosphate, have been implicated as second messengers in the mitogenic actions of certain growth factors (Olivera and Spiegel 1993). The role of sphingolipid metabolites in the regulation of neuronal survival, development and death is now beginning to be appreciated (Riboni et al. 1995). Sphingosylphosphorylcholine (SPC), the N-deacylated derivative of sphingomyelin, is an extremely potent mitogen for a variety of cells (Desai et al. 1993). In some cases, this mitogenic action is triggered by a protein kinase C-dependent pathway (Seufferlein and Rozengurt 1995). SPC has been found to mobilize Ca^{2+} from internal sources by an IP_3-dependent (Okajima and Kondo 1995) and IP_3-independent (Desai et al. 1993, Yule et al. 1993) pathway, and an involvement of ryanodine receptors in SPC-induced Ca^{2+} release from brain microsomes has been reported (Dettbarn et al. 1995, Furuya et al. 1996). Furthermore, a microsomal SPC-gated Ca^{2+} channel in RBL-2H3 cells has been characterized (Kindman et al. 1994, Mao et al. 1996) in addition to the previously described receptor channel activated by IP_3 and ryanodine.

Although sphingolipids are abundant in the central nervous system, the mechanism of these compounds for mobilizing Ca^{2+} from intracellular stores has not been characterized. In the present study, we have investigated the mechanisms of action of SPC and related compounds on Ca^{2+} release in isolated rat synaptosomes.

Synaptosomes were isolated according to Dunkley et al. (1988) with modifications as previously described (Catalán et al. 1996). Isolated synaptosomes were resuspended in Ca^{2+}-free artificial cerebrospinal fluid (CSF) (132 mM NaCl, 3 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 1.2 mM $NaH_2PO_4 \cdot 2H_2O$, 10 mM glucose and 10 mM Hepes, pH was adjusted to 7.4 with Tris base), and washed twice with CSF, centrifuged at 3,600 g for 10 min and resuspended in CSF. To determine the cytosolic Ca^{2+} concentration, synaptosomes (4 mg protein) were loaded with 5 μM Fura 2 AM (Molecular Probes) for 30 min at 37°C in CSF. Incubations were stopped by 10-fold dilution with CSF and suspensions were centrifuged for 10 min at 3,600 g. The pellets were resuspended in CSF (2 mg protein/ml) and in each experiment a synaptosome sample (100 μg protein) was resuspended in 2 ml CSF and placed in a 1 cm^2 quartz cuvette and experiments were performed at 37°C. Changes in Ca^{2+} concentration were determined from dual wavelength measurements at an emission wavelength of 510 nm and excitation wavelength of 340 and 380 nm on a Perkin Elmer Luminescence Spectrometer LS-50. The calcium concentration was calculated according to Grynkiewicz et al. (1985). In some experiments, synaptosomes were permeabilized by incubation with 0.5 IU/ml streptolysin-O in CSF supplemented with 2 mM ATP for 10 min at 20°C, for selective plasma membrane permeabilization. Incubations were stopped by addition of CSF and centrifugation at 3,600 g for 10 min. The pellets were resuspended in an intracellular-like solution containing 20 mM NaCl, 100 mM KCl, 1 mM $MgSO_4$, 20 mM HEPES, 750 nM $CaCl_2$, 1 mM ATP and 1 μM fura-2 pentapotassium salt (pH 7.2) and the Ca^{2+} release was measured in the medium and monitored as described above.

Treatment with doses of SPC from 12.5 μM to 50 μM produced a dose-dependent increase in cytosolic Ca^{2+} concentration in isolated synaptosomes (Fig. 1). When the experiments were performed in the presence of 1 mM external Ca^{2+}, the Ca^{2+} release was instantaneous and was maintained for at least 3.5 min. The greatest effect obtained was with 50 μM SPC (from 215 to 285 nM). A smaller increase was obtained after SPH treatment (Fig. 1). The weaker action of SPH has been proposed as being due to the obligatory phosphorylation of this lipid at the 1-carbon (Ghosh et al. 1994). Our results confirm this hypothesis, in that no effect was observed upon treating synaptosomes with psychosine (PSY), a sphingosine analog, which is galactosylated at the 1-carbon, and thus prevents phosphorylation at that site.

When the experiments were performed in the absence of external Ca^{2+} (in the presence of 2.5 μM EGTA), the effect of SPC was transient and the Ca^{2+} levels reached basal levels after 1 min of treatment (Fig. 2). These findings suggest that the SPC may have a dual action, releasing Ca^{2+} from intracellular stores, and increasing Ca^{2+} entry from the extracellular medium. Sphingolipids have been found to mobilize Ca^{2+} through a modulatory action on the IP_3 pathway (Chao et al. 1994). In addition, SPC induces a substantial release of Ca^{2+} from IP_3-sensitive (Okajima and Kondo 1995) and –insensitive (Desai et al. 1993, Yule et al. 1993) intracellular pools in different cell types. The neurons exhibit at least two pharmacologically and molecularly defined mechanisms by which Ca^{2+} may be released from intracellular compartments:

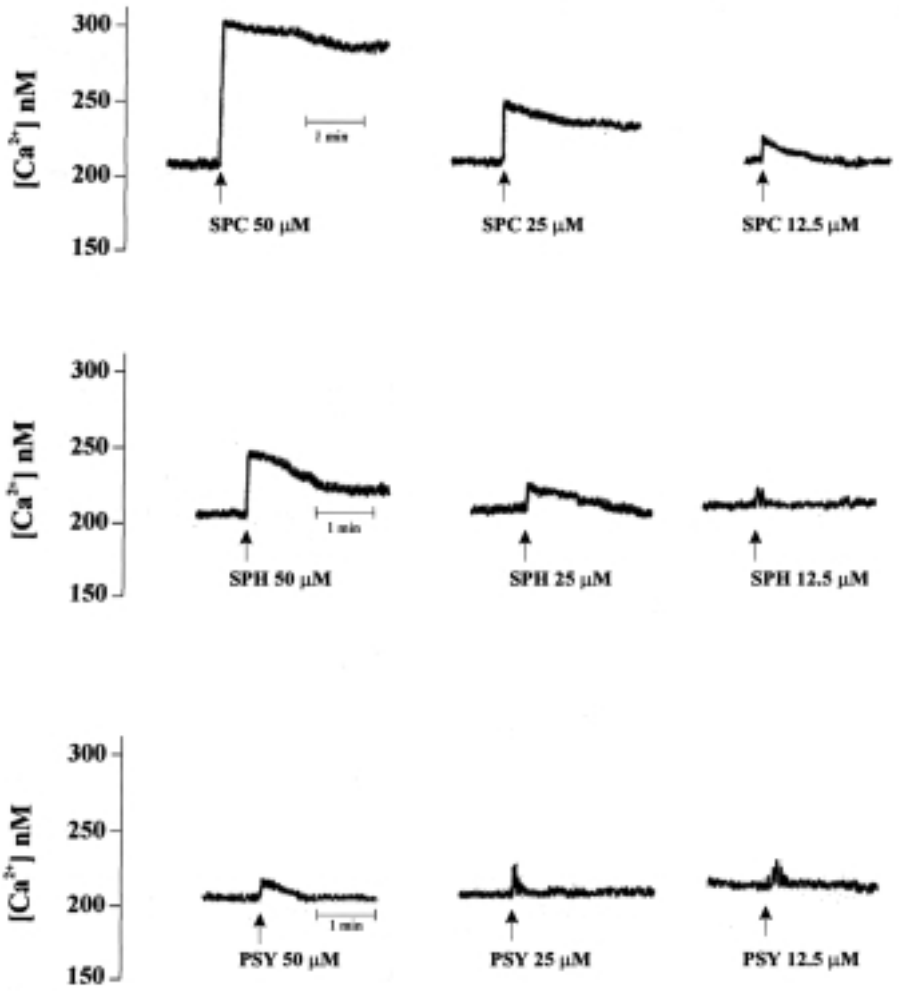

Fig 1. Effect of sphingolipids on Ca^{2+} concentration in isolated synaptosomes. Synaptosomes loaded with Fura 2 were treated with 50 μM sphingosylphosphorylcholine (SPC), sphingosine (SPH) or psychosine (PSY) in a medium containing 1 mM Ca^{2+}. Each trace is representative of at least three experiments.

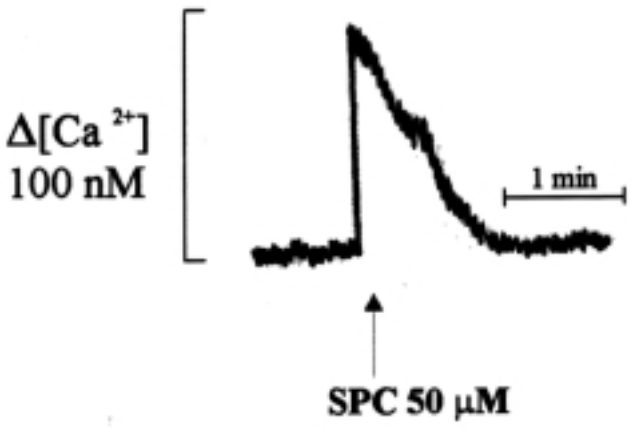

Fig. 2. Sphingosylphosphorylcholine (SPC)-induced increase in Ca^{2+} concentration in isolated synaptosomes incubated in a Ca^{2+}-free medium. Synaptosomes loaded with Fura 2 were incubated with 50 μM SPC in a Ca^{2+}-free medium containing 2.5 μM EGTA. This trace is representative of three experiments.

activation of IP_3 receptors and activation of ryanodine receptors (Burgoyne et al. 1989). To determine whether the SPC-increased Ca^{2+} release reported here occurs by one of these mechanisms, experiments in the presence of heparin, an antagonist of IP_3 receptors, or ryanodine were performed. Results in Fig. 3A indicate that pretreatment of permeabilized synaptosomes with different concentrations of heparin, 2 min prior to the addition of 10 μM IP_3, inhibited the IP_3-induced Ca^{2+} release, in a dose-dependent manner. However, pretreatment with heparin, at concentrations of up to100 μg/ml, did not inhibit the effect produced by SPC. The failure of heparin to block SPC-induced Ca^{2+} release from permeabilized synaptosomes indicates that the Ca^{2+} release does not involve the IP_3 receptor Ca^{2+} channel. In addition, when intact synaptosomes were treated with 200 nM thapsigargin, a microsomal Ca^{2+}-ATPase inhibitor, in the absence of external Ca^{2+}, an increase in cytosolic Ca^{2+} from 215 ± 11 nM to 510 ± 24 nM was observed

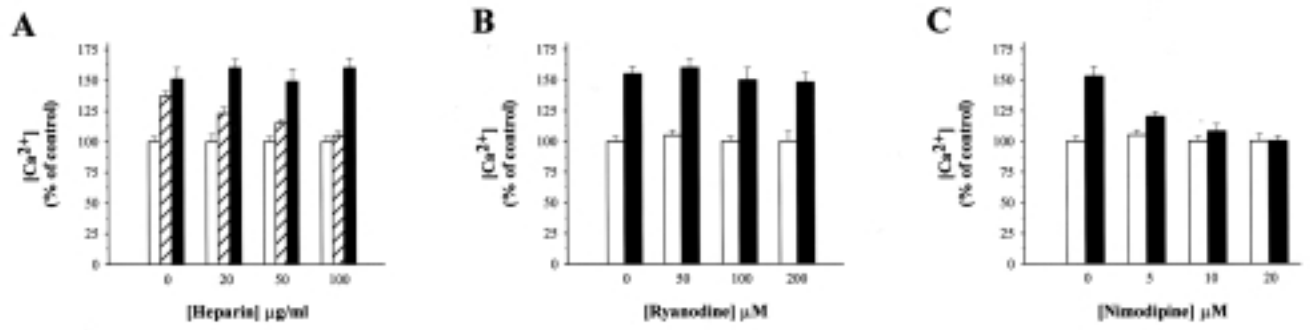

Fig. 3. Effects of several inhibitors on sphingolipid-induced Ca^{2+} release in synaptosomes. Permeabilized synaptosomes were treated with different concentrations of heparin (A) or ryanodine (B). Intact synaptosomes were preincubated with nimodipine (C). In all cases the inhibitors were added 2 min prior to the addition of vehicle (empty bars) or 50 μM SPC (filled bars) or 10 μM IP_3 (stripped bars). Results are given as percentage of changes with respect to basal values (261 ± 13 nM in permeabilized synaptosomes and 215 ± 11 nM in intact synaptosomes). Data are the mean ± SE of three determinations.

(data not shown). The addition of SPC had no effect on thapsigargin-induced Ca^{2+} increase (data not shown), indicating that SPC can mobilize Ca^{2+} from endoplasmic reticulum. Pretreatment with ryanodine (from 50 μM to 200 μM) did not inhibit SPC-induced Ca^{2+} release from synaptosomes (Fig. 3B).

Finally, we investigated the action of nimodipine, an L-type Ca^{2+} channel inhibitor, on SPC-stimulated cytosolic Ca^{2+} concentration in intact synaptosomes. Results indicated that nimodipine, an amphiphilic molecule, that can enter through the membrane (Herbette et al. 1994), prevents the effect of SPC over a concentration range from 5 to 20 μM (Fig. 3C). In this regard, it is known that the blockage of L-type Ca^{2+} channels by nimodipine inhibits changes in intracellular Ca^{2+}concentration evoked by histamine, caffeine and ryanodine. This response is attributed to Ca^{2+} mobilization from intracellular stores (Dessy and Godfraind 1996). In addition, nimodipine caused a depletion of the intracellular Ca^{2+} store in experiments with Ca^{2+}-free medium cultured rat astrocytes (Fischer et al. 1997). Therefore it is possible that nimodipine inhibits both the traffic of Ca^{2+} across the synaptosomal membrane and Ca^{2+} release from internal stores induced by SPC.

We should point out that the existence of an intracellular sphingolipid-gated channel has been proposed (Kindman et al. 1994, Mao et al. 1996). More recently, we have described how sphingosine derivatives increase Ca^{2+} concentration in liver nuclei, possibly by an intracellular sphingosine-sensitive Ca^{2+} channel (Catalán et al. 1997). Taken together, these results suggest that SPC increases intracellular Ca^{2+} through a specific intracellular sphingolipid-gated nimodipine sensitive Ca^{2+} channel.

The present findings suggest a potential role for sphingolipids in the control of the brain functions through regulation of cytosolic Ca^{2+} levels. Further studies are required to establish the physiological role of lysosphingolipids in brain.

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