Pertussis Toxin Inhibits Phospholipase C Activation and Ca\(^{2+}\) Mobilization by Sphingosylphosphorylcholine and Galactosylsphingosine in HL60 Leukemia Cells

IMPLICATIONS OF GTP-BINDING PROTEIN-COUPLED RECEPTORS FOR LYSOSPHINGOLIPIDS*

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Extracellular sphingosylphosphorylcholine (SPC) and galactosylsphingosine (psychosine) induced Ca\(^{2+}\) mobilization in a dose-dependent manner in HL60 leukemia cells. The rapid and transient increase in Intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) elicited by SPC and psychosine at concentrations lower than 30 \(\mu M\) was inhibited by treatment of the cells with pertussis toxin (PTX) and U73122, a phospholipase C inhibitor, as was the case for UTP, a P\(_{2}\)-purinergic agonist. The increase in [Ca\(^{2+}\)]\(_i\), induced by these lysosphingolipids was associated with inositol phosphate production, which was also sensitive to PTX and U73122. The inositol phosphate response is not secondary to the increase in [Ca\(^{2+}\)]\(_i\), as evidenced by the observation that thapsigargin and ionomycin, Ca\(^{2+}\) mobilizing agents, never induced inositol phosphate production and, unlike lysosphingolipids, the [Ca\(^{2+}\)]\(_i\) rise by these agents was totally insensitive to PTX and U73122. When HL60 cells were differentiated into neutrophil-like cells by dibutyryl cyclic AMP, inositol phosphate and Ca\(^{2+}\) responses to AlF\(_4^–\) were enhanced, probably reflecting an increase in the amount of G\(_{12}\) and G\(_{13}\) compared with undifferentiated cells. In the neutrophil-like cells, however, the responses to SPC and psychosine were markedly attenuated. This may exclude the possibility that the lysosphingolipids activate rather directly PTX-sensitive GTP-binding proteins or the phospholipase C itself. Other lysosphingolipids including glucosylsphingosine (glucopsychosine) and sphingosylgalactosyl sulfate (lyso sulphatides) at 30 \(\mu M\) or lower concentrations also showed PTX- and U73122-sensitive Ca\(^{2+}\) mobilization and inositol phosphate response in a way similar to SPC and psychosine. However, platelet-activating factor and lysoglycerophospholipids such as lysophosphatidylcholine and lysophosphatidic acid were less effective than these lysosphingolipids in the induction of Ca\(^{2+}\) mobilization. Taken together, the results indicate that a group of lysosphingolipids at appropriate doses induces Ca\(^{2+}\) mobilization through inositol phosphate production by phospholipase C activation. The lysosphingolipids-induced enzyme activation may be mediated by PTX-sensitive GTP-binding protein-coupled receptors, which may be different from previously identified platelet-activating factor receptor or lysophosphatic acid receptor.

Sphingolipids have recently been shown to be important participants in the regulation of a variety of cellular processes (1–3). Sphingosine, one of the metabolites of sphingolipids, was in its early studies demonstrated as a potent endogenous inhibitor of protein kinase C (1, 4) and has been implicated to be a negative regulator for a few signaling processes (1, 4). Further studies, however, revealed that the exogenous sphingosine also induces various types of positive biological actions, e.g. activation of phospholipase D (5), stimulation of cell proliferation (6), regulation of Ca\(^{2+}\) mobilization from the internal pool (7–11), and inhibition of Ca\(^{2+}\) influx through the plasma membranes (12). These actions seem to be exerted through phosphatidate (5) or a phosphorylated product of sphingosine, sphingosine 1-phosphate (S1P)\(^1\) (7, 8, 13–15); many of them were suggested to be independent of protein kinase C. S1P was reported to act directly on the internal Ca\(^{2+}\) pool resulting in Ca\(^{2+}\) mobilization in a way similar to inositol 1,4,5-trisphosphate (8, 15). This lysosphingolipid has also been proposed as a second messenger of platelet-derived growth factor and serum on cell proliferation in fibroblasts (16). In the brain and other peripheral tissues of inherited sphingolipid disorders, it has been shown that any one of lysosphingolipids, e.g. sphingo- phosphorylcholine (SPC), galactosylsphingosine (psychosine), or glucosylphosphingosine (glucopsychosine), is accumulated (4, 17–19). These lysosphingolipids might be responsible for the respective pathogenesis (4, 17–19). SPC has recently been shown, similarly to S1P, to be a potent Ca\(^{2+}\) releaser from the internal pool and suggested to cause the Ca\(^{2+}\) release from the 1,4,5-trisphosphate-sensitive pool in various cell types (7–10).

These observations suggest that, in addition to protein kinase C inhibition, intracellular Ca\(^{2+}\) mobilization is an important action of lysosphingolipids, which may have pathological and physiological significance. This raises the question of whether the Ca\(^{2+}\) mobilization is caused by the activation of the phospholipase C-Ca\(^{2+}\) signal transduction pathway. In fact, recent studies demonstrated that extracellular S1P in Swiss 3T3 fibroblasts (15) and sphingosine in Swiss 3T3 fibroblasts (5), astrocytes (20), and foreskin fibroblasts (21) can induce inositol phosphate production, probably reflecting activation of phospholipase C. Although the S1P-induced [Ca\(^{2+}\)]\(_i\) increase in the cells has been suggested to occur independently of the enzyme activation (15), at least a part of the sphingosine-induced Ca\(^{2+}\) mobilization as well as phospholipase C activation in foreskin fibroblasts was sensitive to PTX, showing some

* The abbreviations used are: S1P, sphingosine 1-phosphate; PTX, pertussis toxin; [Ca\(^{2+}\)]\(_i\), cytoplasmic free Ca\(^{2+}\) concentration; SPC, sphingosylphosphorylcholine; G-protein, GTP-binding regulatory protein; IP\(_1\), inositol monophosphate; IP\(_2\), inositol bisphosphate; IP\(_3\), inositol trisphosphate.

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similarly to a typical feature of PTX-sensitive G-protein-mediated activation of the phospholipase C-Ca\(^{2+}\) pathway (21). If this is the case, we might be allowed to imagine the presence of a receptor(s) for the lysosphingolipids which lead to the activation of phospholipase C, although the previous findings have not excluded the possibility that the lipids penetrate into the cells and act on the pathway inside the cells.

In the present paper, our study was focussed on the Ca\(^{2+}\) mobilizing actions of SPC and other lysosphingolipids which are accumulated in the respective sphingolipidoses, especially on the mechanisms of their actions. We found that, in HL60 leukemia cells, extracellularly added lysosphingolipids at 30 \(\mu\)M or less induced a rapid and transient increase in [Ca\(^{2+}\)], the features of which are indistinguishable from those of the Ca\(^{2+}\) response induced by UTP, a P\(_2\)-purinergic agonist, in the same cells. The transient [Ca\(^{2+}\)]\(_i\) rise was associated with inositol phosphate production, and both Ca\(^{2+}\) and inositol phosphate responses were inhibited by the treatments of cells with PTX and U73122, a potent phospholipase C inhibitor. Our results suggest that extracellular lysosphingolipids at appropriate doses induce a [Ca\(^{2+}\)]\(_i\) rise due to the activation of the phospholipase C being mediated by a putative receptor(s) coupled to a PTX-sensitive G-protein(s).

**EXPERIMENTAL PROCEDURES**

**Materials**—Sphingosylphosphorylcholine (SPC), 1-\(\beta\)-galactosylsphingosine (psychosine), 1-\(\beta\)-glucosylsphingosine (glucospsychosine), sphingosylgalactosylsulfate (lysosulfatides), sphingosine, thapsigargin, formyl-Met-Leu-Pheng, adenosine deaminase, 1-oleoyl-sn-glycero-3-phosphate (lysophosphatidic acid), lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylinositol, lysophosphatidyserine, sphingomyelin from Bacillus cereus (S-9396), and platelet-activating factor were purchased from Sigma; Fura-2/AM from Dojindo (Tokyo); and [\(^{3}H\)]inositol (23-29). D-[\(^{1}H\)]-glucosylsphingosine (glucopsychosine), 1-\(\beta\)-D-glucosylsphingosine, and lysosulfatides. The purity of the lipids was verified by the “certificate of analysis” of the manufacturer, the purity of lysosphingolipids was checked with a thin-layer chromatogram (23). Gi2 and Gi3 subunits were obtained from the Tokyo Institute of Physical and Chemical Research (RIKEN) (Wako, Japan) and Upjohn Co. (Kalamazoo, MI), respectively. The sources of all other reagents were the same as described previously (23-29).

**Purity Check and Purification of SPC and Psychosine**—According to the “certificate of analysis” of the manufacturer, the purity of sphingolipids is more than 85% for SPC and more than 95% for psychosine, glucospsychosine, and lysosulfatides. The purity of the lipids was checked in the present study by silica Gel 60 (Merck) TLC using two solvent systems (solvent I, butanol/water/acidic acid, 3:1:1 (v/v); solvent II, CHCl3/MEOH/water/acidic acid, 30:30:3:5 (v/v)) (14). In solvent I and II, \(R_s\) values were 0.12 and 0.04 for SPC, 0.39 and 0.62 for psychosine, 0.42 and 0.65 for glucopsychosine, and 0.37 and 0.70 for lysosulfatides, respectively. These lipids were detected with ninhydrin (all samples), molybdenum blue (SPC), and aniline-\(\text{H}_2\)SO\(_4\) (psychosine, glucospsychosine, and lysosulfatides) (14, 30). In the case of the psychosine, glucopsychosine, and lysosulfatides, single spot was detected that was positive with ninhydrin and aniline-\(\text{H}_2\)SO\(_4\) on TLC with either solvent. In the case of the SPC sample, however, there was a trace of unknown spot that was positive with ninhydrin, but not with molybdenum blue, at \(R_s\) of 0.26 in solvent I and 0.20 in solvent II. Since the unknown compound does not seem to affect Ca\(^{2+}\) response, we considered that the above lipids were used in the present study without further purification unless otherwise stated. In some experiments in Fig. 2, SPC and psychosine were purified by silica Gel 60 TLC using solvent I. The region corresponding to SPC or psychosine was scraped off and extracted with CHCl3/MEOH/water (10:10:1) for SPC and with MEOH for psychosine. SPC and psychosine were quantified by the method (31) and the aniline-\(\text{H}_2\)SO\(_4\) method (30), respectively. The TLC-purified lipids were checked for the ability to induce Ca\(^{2+}\) mobilization.

**Cell Cultures**—HL60 cells were routinely cultured in a RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum (Life Technologies, Inc.) and maintained in a humidified atmosphere of 95% air and 5% CO\(_2\). In some experiments in Fig. 7, the cells were cultured for 5 days in a medium containing 50 \(\mu\)M dibutyryl cyclic AMP to differentiate into neutrophil-like cells. Two days before the experiments, the cells were sedimentsed (250 \times g for 5 min) and transferred to fresh medium for [Ca\(^{2+}\)]\(_i\) measurement and membrane preparation. For inositol phosphate response, the cells were transferred to an inositol-free RPMI 1640 medium containing 10% fetal calf serum and myo-[\(^{2}H\)]inositol (4 \(\mu\)Ci/ml). PTX treatment of the cells was performed by adding the toxin (50 ng/ml) to the medium 4 h before the experiments.

Measurement of [\(^{3}H\)]inositol Phosphorylation—The [\(^{3}H\)]inositol-labeled cells were washed by sedimentation (250 \times g for 5 min) and resuspended with Hepes-buffered medium which consisted of 20 mM Hepes (pH 7.5), 134 mM NaCl, 4.7 mM KCl, 1.2 mM KH\(_2\)PO\(_4\), 1.2 mM MgSO\(_4\), 2 mM CaCl\(_2\), 2.5 mM NaHCO\(_3\), 5 mM glucose, and 0.1% (w/v) bovine serum albumin (fraction V). The washing procedure was repeated, and the cells were finally resuspended in the same medium. The cells (about 2 \times 10\(^5\) cells) were preincubated for 10 min with 10 mM LiCl and 0.5 units/ml adenosine deaminase in polypropylene vials (20 ml) in a final volume of 1.5 ml. The test agents (>100) were then added to the medium and incubated for 1 min unless otherwise specified. The cell suspension (0.5 ml) in duplicate was transferred to tubes containing 1 ml of CHCl3/MEOH/HCl (200:100:1). [\(^{3}H\)]inositol mono-, di-, and triphosphates were separated as described previously (27). The radioactivity of the trichloroacetic acid (5%)-insoluble fraction was measured as the total radioactivity incorporated into the cellular inositol lipids. Where indicated, the results were normalized to 10\(^5\) cpm of the total radioactivity.

**Measurement of [Ca\(^{2+}\)]\(_i\)**—The cells were sedimented, resuspended in HL60 medium containing 0.1% bovine serum albumin, and then incubated for 20 min with 1 \(\mu\)M Fura-2/AM. [Ca\(^{2+}\)]\(_i\) was estimated from the change in the fluorescence of the Fura-2-loaded cells as described previously (27, 29).

**Measurement of Phospholipase C Activation by Lysosphingolipids**—In the present paper, our study was focussed on the Ca\(^{2+}\) mobilizing actions of SPC and other lysosphingolipids which are accumulated in the respective sphingolipidoses, especially on the mechanisms of their actions. We found that, in HL60 leukemia cells, extracellularly added lysosphingolipids at 30 \(\mu\)M or less induced a rapid and transient increase in [Ca\(^{2+}\)], the features of which are indistinguishable from those of the Ca\(^{2+}\) response induced by UTP, a P\(_2\)-purinergic agonist, in the same cells. The transient [Ca\(^{2+}\)]\(_i\) rise was associated with inositol phosphate production, and both Ca\(^{2+}\) and inositol phosphate responses were inhibited by the treatments of cells with PTX and U73122, a potent phospholipase C inhibitor. Our results suggest that extracellular lysosphingolipids at appropriate doses induce a [Ca\(^{2+}\)]\(_i\) rise due to the activation of the phospholipase C being mediated by a putative receptor(s) coupled to a PTX-sensitive G-protein(s).
These results suggest that at least the PTX-sensitive increase in \([\text{Ca}^{2+}]_i\) by SPC and psychosine is independent of the protein kinase C inhibition.

TLC analysis of the SPC sample showed the presence of a small, but detectable, amount of unknown compound that is positive with ninhydrin at \(R_f = 0.26\) (Fig. 2A, lane 1). However, it was confirmed that SPC itself elicited the \([\text{Ca}^{2+}]_i\) response and the contaminated unknown compound is inactive to induce the response (Fig. 2). Sphingomyelinase from \(B.\) cereus almost completely converted SPC to sphingosine, but did not influence the unknown compound (Fig. 2A, lanes 2–4). The enzyme-treated SPC never elicited a rapid and transient \([\text{Ca}^{2+}]_i\) increase which is characteristic to the untreated SPC, instead induced a rather slow increase probably due to sphingosine (Fig. 2C). The enzyme was rather specific to SPC; psychosine was tolerable to the enzyme (Fig. 2A, lanes 6 and 7) and the lipid-induced \([\text{Ca}^{2+}]_i\) increase was unaffected by its treatment (Fig. 2C).

Furthermore, the TLC-purified SPC sample, which is free from the unknown compound (Fig. 2A, lane 5), induced the \([\text{Ca}^{2+}]_i\) response to an extent similar to that of the unpurified SPC (Fig. 2C).

Although psychosine obtained from the drug company showed a single spot that is positive with ninhydrin and anthrone-\(\text{H}_2\text{SO}_4\) on TLC using two solvent systems, we further purified the psychosine by TLC (Fig. 2B, lanes 1 and 2). The purified psychosine also induced a rapid and transient \([\text{Ca}^{2+}]_i\) increase as effectively as the unpurified psychosine did (Fig. 2D). Since the active compound to induce \([\text{Ca}^{2+}]_i\) mobilization was demonstrated to be SPC or psychosine itself and furthermore, there was no appreciable difference in the ability to...
 induce Ca\(^{2+}\) response between purified and unpurified products, we performed the following experiments without further purification of the lipids.

SPC and Psychosine Mobilize Ca\(^{2+}\) from the Internal Pool in a Manner Sensitive to U73122, a Phospholipase C Inhibitor—As shown in Fig. 3, an addition of excess EGTA to the incubation medium hardly affected the [Ca\(^{2+}\)]\(\text{c}\) increase due to SPC, psychosine, and UTP at 30 \(\mu M\), 30 \(\mu M\), and 1 \(\mu M\), respectively. These results suggest that the increased [Ca\(^{2+}\)]\(\text{c}\) induced by these lysosphingolipids is derived predominantly from intracellular pools. Although this is not inconsistent with the recent observations where SPC mobilizes Ca\(^{2+}\) by rather direct interactions with intracellular pools in DDT\(\_\)MF-2 smooth muscle cells (7, 8), pancreatic acinar cells (9), and basophilic leukemia cells (10), the present finding of the similarity of the Ca\(^{2+}\) response pattern to the UTP actions also suggests that the Ca\(^{2+}\) mobilization by these lysosphingolipids is caused by the activation of the phospholipase C-Ca\(^{2+}\) signaling pathway.

In favor of the latter suggestion, U73122, a potent phospholipase C inhibitor (33) completely inhibited the SPC effect at 30 \(\mu M\) (Fig. 3A). The situation was similar for psychosine, although the 30 \(\mu M\) psychosine effect was not completely abolished by the phospholipase C inhibitor (Fig. 3B). Under these conditions, the UTP effect was totally sensitive to U73122 (Fig. 3C). These results suggest that SPC and psychosine at 30 \(\mu M\) induce Ca\(^{2+}\) mobilization predominantly through phospholipase C activation.

SPC and Psychosine Produce Inositol Phosphate—Fig. 4, A, C, and E, show that 30 \(\mu M\) SPC and psychosine induced inositol phosphate production, which may reflect activation of phospholipase C. The time courses of the production of three species of inositol phosphate induced by both SPC and psychosine were very similar to those by UTP which activates the enzyme through a P2-receptor (32). The actions of the lysosphingolipids as well as UTP were markedly inhibited by a PTX treatment (Fig. 4, B, D, and F), suggesting the involvement of a PTX-sensitive G-protein(s) in the lysosphingolipid-induced phospholipase C activation. The PTX treatment suppressed more than 70% of the lipid-induced activation at any dose (Fig. 5, A and B). As shown in this figure, U73122 markedly inhibited the inositol phosphate production, confirming that these lysosphingolipid actions are due to the activation of phospholipase C.

PTX and U73122-sensitive Activation of Phospholipase C Is not Secondary to the [Ca\(^{2+}\)]\(\text{c}\) Rise—The actions of lysosphingolipids, as shown in the previous section, bear characteristics of the activation of the phospholipase C-Ca\(^{2+}\) pathway through receptors coupling to PTX-sensitive G-proteins. On the other hand, another possibility remains that might explain the events in a reverse way, that is, a Ca\(^{2+}\)-induced phospholipase C activation, because previous studies have demonstrated phospholipase C activation by increased [Ca\(^{2+}\)]\(\text{c}\) (34) in addition to lysosphingolipid-induced [Ca\(^{2+}\)]\(\text{c}\) increase by their direct action on intracellular Ca\(^{2+}\) pools (7–10). This possibility, however, can be ruled out based on the following observations. In Fig. 6, we examined the effect of ionomycin, a Ca\(^{2+}\)-ionophore, and thapsigargin on the cells. Thapsigargin inhibits Ca\(^{2+}\) uptake into its intracellular pool by inhibiting Ca\(^{2+}\)-ATPase, resulting in an increase in [Ca\(^{2+}\)]\(\text{c}\). Both agents increased [Ca\(^{2+}\)]\(\text{c}\)
to an extent similar to 30 μM SPC and psychosine. The Ca2+ increase by these agents, however, was hardly modified by the treatments of the cells with U73122 and PTX (Fig. 6, A and B). Moreover, inositol phosphate was not significantly produced by the incubation of the cells with these Ca2+ mobilizers for at least 5 min, while in the same experiment, an appreciable production of inositol phosphate was found in the presence of SPC at 30 μM (Fig. 6C).

Differentiation into Neutrophil-like Cells Was Associated with Attenuation of Responses to Lysosphingolipids—HL60 cells can be differentiated into neutrophil-like cells by treatment of the cells with dibutyryl cyclic AMP or other inducers. Cells differentiated with formyl-Met-Leu-Phe-induced [Ca2+]i increase (Fig. 7A). The content of Gi2 and Gi3 was actually evidenced from increases in immunodetectable Gi2 and Gi3 in HL60 cells treated with dibutyryl cyclic AMP and other inducers. Increase in PTX-sensitive G-proteins, Gi2 and Gi3, is accompanied by differentiation (32, 35). Because the foregoing results suggest an involvement of the toxin-sensitive G-proteins in the lysosphingolipid signaling, the cell differentiation would potentiate the actions of lysosphingolipids.

As shown in Fig. 7A, the contents of Gi2 and Gi3 were actually increased by a dibutyryl cyclic AMP treatment of the cells as evidenced from increases in immunodetectable Gi2 and Gi3. The dibutyryl cyclic AMP-treated cells also showed a PTX-sensitive formyl-Met-Leu-Phe-induced [Ca2+]i increase (Fig. 7C) and inositol phosphate production (Fig. 7E). In contrast, the subsequent Ca2+ mobilization in many types of cells is slow and significant, and are slightly stronger in the differentiated cells than in the undifferentiated ones, probably reflecting higher contents of G-proteins in the neutrophil-like differentiated cells than in the undifferentiated cells (Fig. 7D and E). Unexpectedly, however, the SPC-induced Ca2+ mobilization was markedly attenuated in the neutrophil-like cells (Fig. 7B and C). The Ca2+ response to psychosine was also decreased (Fig. 7D). In parallel with the Ca2+ response, the inositol phosphate response to SPC and psychosine was clearly attenuated by differentiation, suggesting that the lipids signaling of the PTX-sensitive G-protein-coupled phospholipase C-Ca2+ pathway is blocked before a G-protein step in the neutrophil-like cells (Fig. 7E).

Glucopsinosine and Lysosulfatides Also Induce Ca2+ Mobilization and Inositol Phosphate Production—We next examined the effects of glucopsinosine and lysosulfatides, which have been suggested to be accumulated in other sphingolipidosises, i.e. Gaucher’s disease and metachromatic leukodystrophy, respectively (4, 17–19). Results (IP2 production) are expressed as percentages of initial values. Data are means ± S.E. of three separate experiments.
Phospholipase C Activation by Lysosphingolipids

increase in \([Ca^{2+}]\), was followed by the sustained increase. The early transient rises at 10 and 30 \(\mu M\) lipids were markedly suppressed by U73122 and PTX treatment, while the later sustained increase was rather resistant to these agents. The U73122-insensitive \([Ca^{2+}]\) increase was also detected in the presence of 2.5 mM EGTA (data not shown), suggesting that the source of \([Ca^{2+}]\) is the internal pool. However, because it is also possible that the lipids induced the leakage of the fluorescence indicator, Fura-2, we cannot conclude that the U73122-insensitive phospholipase C activation. In fact, as shown in Fig. 8C, a significant inositol phosphate production was observed immediately after the addition of either lysosphingolipid, although the lysosulfatides effect was diminished after 1 min. The inositol phosphate production was also abolished by PTX treatment (Fig. 8D). Thus these two lyso compounds caused essentially the same responses in the cells as those induced by SPC and psychosine.

Lysosphingolipids and Platelet-activating factor Also Induce \(Ca^{2+}\) Mobilization, but They Were Less Effective Than Lysosphingolipids—We also examined the effect of sphingomyelin and galactosylceramide on \(Ca^{2+}\) mobilization. These lipids are derivatives of SPC and psychosine, respectively, and each having a fatty acyl moiety linked to their amino group. However, these sphingolipids hardly influenced the \([Ca^{2+}]\) level, confirming again that the SPC and psychosine effects on the \([Ca^{2+}]\) level is not due to the possible contamination of the precursor molecules (Table I). Some of glycerophospholipids and lysosphingolipids, such as platelet-activating factor and lysophosphatidic acid, have already been shown to induce a variety of biological responses including \(Ca^{2+}\) mobilization in many types of cells (37, 38). As shown in Table I, platelet-activating factor and some of lysosphingolipids also induced significant \(Ca^{2+}\) mobilization, but none of them was as potent as SPC and other lysosphingolipids. PTX treatment was also inhibitory for their action except for the lysophosphatidic acid-induced one; the \(Ca^{2+}\) mobilization induced by lysophosphatidic acid was hardly affected by toxin treatment (Table I). In contrast to SPC and psychosine action (Fig. 7), platelet-activating factor-induced \(Ca^{2+}\) response was markedly enhanced, but not attenuated, by dibutyryl cAMP-induced differentiation; net \([Ca^{2+}]\) increase by platelet-activating factor at

2\footnote{We should be cautious to interpret the fluorescence data in the case of the sustained pattern in our experimental conditions; it is possible that the amphipathic lipids induced the leakage of the fluorescence indicator. In fact, when we employed 300 \(\mu M\) SPC, the fluorescence intensity increased rapidly to the level near the maximal intensity even in the presence of 2.5 mM EGTA and its high level was sustained, where we found that the fluorescence indicator leaked into the medium. In contrast, in the case of the transient fluorescence change, the leakage of the indicator may not be always necessary to consider, because it is implausible that the leaky cells reuptake the leaked indicator. In addition, the early transient fluorescence changes by the lysosphingolipids shown in the present study were inhibitable by agents such as U73122 and PTX, rather specific agents for phospholipase C and G, proteins, respectively.}
10 μM in the differentiated cells was 708 ± 60% of that in the undifferentiated control cells (number of observations was 3).

DISCUSSION

In the present paper we have shown that lysosphingolipids (SPC, psychosine, glucopsychosine, and lysosulfatides) at doses lower than 30 μM induce phospholipase C activation and the subsequent Ca\(^{2+}\) mobilization in a manner sensitive to PTX and U73122, a phospholipase C inhibitor. This is, to our knowledge, the first indication that these lysosphingolipids activate the phospholipase C-Ca\(^{2+}\) system possibly through receptors coupling to a PTX-sensitive G-protein(s). The putative receptors may be different from the previously identified platelet-activating factor receptor (37) and lysophosphatidic acid receptor (38).

As far as extracellular SPC-induced intracellular Ca\(^{2+}\) mobilization is concerned, a few studies on fibroblasts (39) and FRTL-5 thyroid cells (40) have been reported. However, no significant production of inositol phosphate was observed in these experiments (39, 40), despite the fact that Ca\(^{2+}\) mobilizing receptor agonists, such as bradykinin, induced not only Ca\(^{2+}\) mobilization to an extent similar to that with SPC but also phospholipase C activation under the same conditions (39). In addition, in other studies, SPC mobilized Ca\(^{2+}\) from permeabilized cells (7-10) and purified endoplasmic reticulum membrane vesicles (8). On the basis of these previous results, the SPC actions have been currently considered to occur inside the cells by the incorporated SPC molecules, without activating phospholipase C. The present results, however, suggest that at least the early phase of the Ca\(^{2+}\) mobilization induced by lower than 30 μM SPC or other lysosphingolipids in intact HL60 cells

### TABLE I

| Stimulus                  | [Ca\(^{2+}\)] (nM) | Δ[Ca\(^{2+}\)] % of response to SPC |
|---------------------------|--------------------|-------------------------------------|
| **Lysosphingolipids**     |                    |                                     |
| SPC                       | 100                | 31 ± 7                              |
| Psychosine                | 61 ± 13            | 13 ± 2                              |
| Glucopsychosine           | 57 ± 4             | 15 ± 1                              |
| Lysosulfatides            | 49 ± 3             | 11 ± 1                              |
| **Sphingolipids**         |                    |                                     |
| Sphingomyelin             | 8                  | ND*                                 |
| Galactosylceramide        | 5                  | ND*                                 |
| **Platelet-activating factor** |            |                                     |
|                            | 33 ± 2             | 8 ± 1                               |
| **Lysoglycerophospholipids** |                |                                     |
| Lysocephatidylcholine     | 34 ± 4             | 12 ± 3                              |
| Lysocephatidylethanolamine| 31 ± 4             | 11 ± 2                              |
| Lysocephatidyinositol     | 22 ± 5             | 10 ± 4                              |
| Lysocephatidylserine      | 5 ± 2              | ND*                                 |
| Lysocephatidic acid       | 23 ± 5             | 21 ± 4                              |

* ND, not determined.
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is mediated by the activation of the enzyme. This suggestion is based on the following findings. First, SPC and other lysosphingolipids at doses lower than 30 μM induced immediate activation of phospholipase C. Second, U73122, a potent phospholipase C inhibitor, suppressed at least the early phase of the lysosphingolipids-induced increase in [Ca\(^{2+}\)]\(_i\). Third, treatment of the cells with either PTX or dibutyryl cyclic AMP attenuated both the lysosphingolipid-induced phospholipase C activation and Ca\(^{2+}\) mobilization. Finally, phospholipase C activation is not a secondary response to the increase in [Ca\(^{2+}\)]\(_i\), agents such as thapsigargin and ionomycin, which primarily increase [Ca\(^{2+}\)]\(_i\), never activated the enzyme in HL60 cells under the present conditions (Fig. 6).

Several findings in the present study suggest that the lysosphingolipids signaling is performed through G-protein-coupled receptors. The pattern and kinetics of [Ca\(^{2+}\)]\(_i\) increase and inositol phosphate production by the lysosphingolipids were very similar to those of the responses to a G-protein-coupled receptor agonist, UTP (a P2U-purinergic agonist) (Figs. 1, 3, and 4). Furthermore, as stronger evidence for the involvement of G-protein coupled receptors, the lysosphingolipid actions are suppressed by prior treatment of the cells with PTX which, as is well known, ADP-ribosylates G-proteins and thereby blocks communication between receptors and effector enzymes. Similar PTX sensitivity has already been shown in the phospholipase C activation induced by several receptor agonists such as formyl-Met-Leu-Phe and UTP in leukocytes such as HL60 cells and neutrophils. This finding has been concluded to reflect the fact that receptors coupling to PTX-sensitive G-proteins mediate the phospholipase C activation (24–26, 32). In this analogy, it is reasonable to assume that the lysosphingolipid actions are mediated via G-protein-coupled receptors. It is still possible, however, that amphiphatic lysosphingolipids penetrate into the cells and then directly activate G-proteins. If this was the case, PTX would block the lipid-induced actions. This possibility is excluded from the experiments shown in Fig. 7. Dibutyryl cyclic AMP-induced differentiation into neutrophil-like cells enhanced ALF\(_2\) (a nonspecific G-protein activator)-induced phospholipase C activation, probably reflecting the increase in the amount of G-proteins. This suggests that in the differentiated cells, the downstream region of the G-protein-mediated signaling cascade leading to phospholipase C activation and Ca\(^{2+}\) mobilization is rather fortified by the increase in PTX-sensitive G-proteins. On the contrary, the SPC and psychosine-induced enzyme activation was seriously suppressed by differentiation of the cells. This suggests that differentiation impairs the process between the action sites of lipids (or receptors) and G-proteins and hence may rule out the possibility that these lysosphingolipids directly activate G-proteins. Thus, the present pharmacological study suggests the existence of G-protein-coupled receptors for lysosphingolipids, although conclusive evidence for the existence of the receptors will have to await their molecular cloning.

In addition to lysosphingolipids, platelet-activating factor and some lysoglycerophospholipids, such as lysophosphatidylcholine and lysophosphatidic acid, also induced Ca\(^{2+}\) mobilization in HL60 cells, but they were not as effective as SPC and other lysosphingolipids (Table I). Furthermore, in contrast to SPC and psychosine effects which were attenuated in dibutyryl cAMP-induced differentiated cells (Fig. 7), platelet-activating factor-induced response was conversely enhanced by the induction of differentiation, suggesting that the putative receptors for lysosphingolipids are different from platelet-activating factor receptor. Among lysoglycerophospholipids examined, lysophosphatidylcholine was the most effective in the induction of Ca\(^{2+}\) mobilization (Table I). Similarly to the actions of lysosphingolipids, the lysophosphatidylcholine effect was PTX-sensitive, whereas the lysophosphatidic acid-induced response was not (Table I). Thus, the receptor for lysophosphatidic acid (38) appears to be different from putative receptors for lysosphingolipids. On the other hand, it remains unclear whether lysosphingolipids (including lysophosphatidylcholine, lysophosphatidylethanolamine, and lysophosphatidylinositol) other than lysophosphatidic acid share with lysosphingolipids the same receptor and signaling pathways.

Among lysosphingolipids, sphingosine and S1P have been previously shown to induce phospholipase C activation and the Ca\(^{2+}\) mobilization in a few types of cells (5, 15, 20, 21). In HL60 cells, sphingosine induced the Ca\(^{2+}\) mobilization; however, this action was PTX-insensitive (Fig. 1). Furthermore, the [Ca\(^{2+}\)]\(_i\), increase due to the lipid was so slow that it took 1-3 min to reach a peak value (Fig. 1). Thus, the sphingosine signaling pathway seems to be different from that of SPC and other lysosphingolipids. This also suggests that the PTX-sensitive Ca\(^{2+}\) mobilization by lysosphingolipids cannot be explained by the inhibition of protein kinase C, because sphingosine is a protein kinase C inhibitor similar to or more potent than the lysosphingolipids examined in the present study (4). We also preliminarily examined S1P actions on phospholipase C and the Ca\(^{2+}\) mobilization in HL60 cells. This lipid also activated the enzyme and increased [Ca\(^{2+}\)]\(_i\), in the cells. In this case, we could not detect any difference between S1P and SPC actions in their sensitivity to PTX and U73122. Thus, S1P seems to play a signaling pathway similar to that of SPC in HL60 cells. In Xenopus oocytes, however, S1P activated a Ca\(^{2+}\) channel probably through phospholipase C activation, but SPC could not mimic the S1P action (41). The receptor cloning again would make it clear whether all the lysosphingolipids and some lysoglycerophospholipids share the same receptor or each lipid interacts with its own receptor.

At the present stage of investigation, the physiological roles of the lysosphingolipid-induced activation of the phospholipase C-Ca\(^{2+}\) pathway in leukocytes have not been clarified yet. This type of lysosphingolipid signaling was attenuated by dibutyryl cyclic AMP-induced differentiation of HL60 cells into neutrophil-like cells (Fig. 7). In the preliminary experiments, we found that other differentiation inducers such as dimethyl sulfoxide, retinoic acid, and vitamin D\(_2\) also diminished such lysosphingolipid signaling. This may suggest that only under undifferentiated conditions lysosphingolipids act as physiological and extracellular signals which are oriented to the phospholipase C-Ca\(^{2+}\) pathway. In the previous study in differentiated cells such as fibroblasts (39, 42) and thyroid cells (40), SPC has been shown to be a potent mitogen. The Ca\(^{2+}\) mobilizing action of SPC may be involved in the cell proliferation (39, 40). A preliminary finding in the undifferentiated HL60 cells, however, showed that SPC rather attenuated the cell growth and instead facilitated cell attachment to culture dishes. This phenomenon might reflect a physiological role of SPC as an inducer of cell differentiation. Further study is now in progress to clarify this point.

The possible existence of cell surface receptors for lysosphingolipids may allow consideration of a novel autocrine or paracrine regulatory mechanism operated by the lysosphingolipids in a way similar to other lipid mediators such as prostaglandins and leukotriens. At present, there are no data on the extracellular occurrence of lysosphingolipids in vivo. To establish the autocrine or paracrine role of the lipids, further studies on the problems are needed, which include characterization of intracellular and extracellular metabolic pathways and physiological functions of the lysosphingolipids as well as identification of their putative receptors.
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