Formyl Peptide Receptor Signaling in HL-60 Cells through Sphingosine Kinase*

(Received for publication, July 22, 1998, and in revised form, November 23, 1998)

Regina Alemany, Dagmar Meyer zu Heringdorf, Chris J. van Koppen, and Karl H. Jakobs‡

From the Institut für Pharmakologie, Universitätsklinikum Essen, D-45122 Essen, Germany

Sphingosine-1-phosphate (SPP) produced from sphingo-
sine by sphingosine kinase has recently been reported
to act as intracellular second messenger for a number of
plasma membrane receptors. In the present study, we
investigated whether the sphingosine kinase/SPP path-
way is involved in cellular signaling of the G protein-
coupled formyl peptide receptor in myeloid differenti-
ated human leukemia (HL-60) cells. Receptor activation
resulted in rapid and transient production of SPP by
sphingosine kinase, which was abolished after pertussis
toxin treatment. Direct activation of heterotrimeric G
proteins by AlF₄⁻ also rapidly increased SPP formation
in intact HL-60 cells. In cytosolic preparations of HL-60
cells, sphingosine kinase activity was stimulated by the
stable GTP analog, guanosine 5′-(3-thiotriphosphate).
Inhibition of sphingosine kinase by di-threo-dihy-
droinosphingosine and N,N-dimethylsphingosine did not
affect phospholipase C stimulation and superoxide pro-
duction but markedly inhibited receptor-stimulated Ca²⁺
mobilization and enzyme release. We conclude that
the formyl peptide receptor stimulates through G₁-type
G proteins SPP production by sphingosine kinase, that
the enzyme is also stimulated by direct G protein acti-
vation, and that the sphingosine kinase/SPP pathway
apparently plays an important role in chemotactic
signaling in myeloid differentiated HL-60 cells.

During the last few years, it has become clear that sphingo-
lipids, in addition to being structural constituents of cell mem-
branes, are sources of important signaling molecules. Particu-
larly, the sphingolipid metabolites, ceramide and sphingo-
sine-1-phosphate (SPP),¹ have emerged as a new class of potent
bioactive molecules, implicated in a variety of cellular
processes such as cell differentiation, apoptosis, and proliferation
(1–4). Interest in SPP focused recently on two distinct cellular
actions of this lipid, namely its function as extracellular ligand
activating specific G protein-coupled membrane receptors and
its role as intracellular second messenger (5). Important clues
to a specific intracellular action of SPP were the following
findings. First, activation of various plasma membrane recep-
tors, such as the platelet-derived growth factor receptor (6, 7),
the FceRI (8), and the FcγRI antigen receptors (9), was found to
rapidly increase intracellular SPP production through stimula-
tion of sphingosine kinase. Second, inhibition of sphingosine
kinase stimulation strongly reduced or even prevented cellular
events triggered by these tyrosine kinase-linked receptors,
such as receptor-stimulated DNA synthesis, Ca²⁺ mobilization,
and vesicular trafficking (6, 8, 9). Finally, intracellular SPP
was found to mimic the receptor responses, i.e. it stimulated
DNA synthesis and mobilized Ca²⁺ from internal stores (10–
14). We recently reported that the G protein-coupled musca-
rinic acetylcholine receptor subtypes m2 and m3 expressed in
HEK-293 cells also induce a rapid and transient SPP produc-
tion by sphingosine kinase. Furthermore, intracellular injec-
tion of SPP rapidly mobilized Ca²⁺ in intact HEK-293 cells and
inhibition of sphingosine kinase markedly inhibited Ca²⁺ sig-
naling by these and other G protein-coupled receptors (14).

To characterize the mechanisms leading to sphingosine ki-
nae activation by G protein-coupled receptors and the cellular
role of this pathway, we investigated whether the formyl pep-
tide receptor stimulates sphingosine kinase in myeloid differ-
entiated human leukemia (HL-60) cells, whether G proteins
participate in this process, and, finally, whether sphingosine
kinase activation is involved in specific cellular responses to
receptor stimulation in HL-60 cells. It is well established that
most, if not all, cellular responses to formyl peptide receptors in
HL-60 cells and neutrophils, including phospholipase C (PLC)
stimulation, Ca²⁺ mobilization, superoxide production, and en-
zyme release, are mediated by pertussis toxin (PTX)-sensitive
G₁-type G proteins (15). Here, we report that formyl peptide
receptor activation rapidly stimulates sphingosine kinase in
differentiated HL-60 cells and that G proteins are involved in
this process. Moreover, it is shown that inhibition of sphingo-
line kinase by di-threo-dihydrosphingosine (DHS) or N,N-di-
ethylsphingosine (DMS) does not affect PLC stimulation and
superoxide production but largely inhibits receptor-stimulated
Ca²⁺ mobilization and enzyme release.

EXPERIMENTAL PROCEDURES

Materials—N-Formyl-methionyl-leucyl-phenylalanine (fMLP), lu-
cigenin, dibutyryl cAMP, cytochalasin B, staurosporine, N-nitrophenyl-
β-D-glucosaminide, and N-nitrophenyl-β-D-glucosaminide were obtained
from Sigma. GTPγS and GDPγS were from Boehringer Mannheim.
Myo-[2-3H]inositol (24.4 Ci/mmol) was from NEN Life Science Products,
v-erythro-[3H]sphingosine (15 Ci/mmol) was from ARC, RPMI 1640
medium was from Life Technologies, Inc., bisindolylmaleimide I (8
and GDPγS were from Boehringer Mannheim.
Myo-[2-3H]inositol (24.4 Ci/mmol) was from NEN Life Science Products,
v-erythro-[3H]sphingosine (15 Ci/mmol) was from ARC, RPMI 1640
medium was from Life Technologies, Inc., bisindolylmaleimide I (8
and GDPγS were from Boehringer Mannheim.
Myo-[2-3H]inositol (24.4 Ci/mmol) was from NEN Life Science Products,
supplemented with 10% fetal calf serum, 150 units/ml penicillin, and 150 μg/ml streptomycin in 5% CO₂. Differentiation into neutrophil-like cells was induced by culturing HL-60 cells for 48 h in the presence of 0.5 mM dibutyryl cAMP. For PTX treatment, cells were incubated for 20 h with 50 ng/ml PTX (16, 17).

**Assay of Sphingosine Kinase in Intact HL-60 Cells**—Sphingosine kinase-catalyzed formation of SPP was measured as described before (14). In brief, differentiated HL-60 cells (1.8 × 10⁵ cells/ml) equilibrated in Hanks’ balanced salt solution/bovine serum albumin for 5 min at 37 °C were incubated with [³H]sphingosine (~10⁶ cpm; final concentration, 30 nM) for 30 s to 10 min at 37 °C in a total volume of 200 μl. The reactions were stopped by addition of 2 ml of ice-cold methanol, followed by 1 ml of chloroform. After vigorous vortexing and incubation for 1 h at 37 °C, particulate matter was pelleted by centrifugation, and the supernatant was evaporated to dryness in a SpeedVac centrifuge. After redissolving in 20 μl of methanol, the samples were spotted onto Silica gel 60 TLC plates (Merck), together with authentic unlabeled sphingosine and SPP. Separation of the products was achieved by TLC in 1-butanol:acetic acid:water (3:1:1) as a solvent system. Sphingosine and SPP spots were visualized by staining with ninhydrin spray. After scraping, radioactivity in the spots was measured by liquid scintillation counting.

Formation of [³H]SPP is expressed as cpm/1.8 × 10⁵ cells and corrected for time 0 count rates, amounting to 80–100 cpm.

**Assay of Sphingosine Kinase Activity in HL-60 Cytosol**—Differentiated HL-60 cells were suspended in ice-cold lysis buffer (1.2 × 10⁶ cells/ml) containing 100 mM potassium phosphate buffer (pH 7.4), 1 mM EDTA, 10 mM MgCl₂, 1 mM 2-mercaptoethanol, and disrupted on ice by 50–100 strokes in a pre-cooled Dounce homogenizer. After centrifugation for 20 min at 50,000 g, the supernatant (cytosolic fraction) was collected and used directly in sphingosine kinase assays. Enzyme activity was determined for 20 min at 37 °C in a total volume of 200 μl, containing 10 μM [³H]sphingosine (~10⁶ cpm), 1 mM ATP, 10 mM MgCl₂, 1 mM EDTA, 1 mM 2-mercaptoethanol, 1 mg/ml fatty acid-free bovine serum albumin, 100 mM potassium phosphate buffer (pH 7.4), and HL-60 cytosol (120–200 μg of protein). Reactions were terminated by addition of 2 ml of ice-cold methanol. Extraction, TLC separation, and quantification of [³H]SPP were carried out as described above. Sphingosine kinase activity is expressed as pmol SPP formed per mg protein and 20 min.

**[Ca²⁺]** Measurements—Intracellular free Ca²⁺ concentration ([Ca²⁺]) was determined with the fluorescent Ca²⁺ indicator dye Fura-2 in a Hitachi spectrofluorimeter as described before (18). In some experiments, Ca²⁺ was omitted from the medium, and in addition 5 mM EGTA was added 2 min before stimulus exposure.

**Assay of PLC**—Measurement of PLC-catalyzed inositol phosphate formation was performed as described in detail previously (19). In brief, after labeling for 48 h with myo-[³H]inositol (2.5 μCi/ml) in isoinositol-free medium, differentiated HL-60 cells were equilibrated for 10 min at 37 °C in Hanks’ balanced salt solution/bovine serum albumin with or without the indicated agents. Cells were then incubated with 10 μM fMLP at 37 °C in the presence of 10 mM LiCl for 20 s and 10 min to measure formation of [³H]inositol 1,4,5-trisphosphate (IP₃) and total [³H]inositol phosphates, respectively, as reported before (19).

**Measurement of Superoxide Anion Generation**—Generation of superoxide anions in differentiated HL-60 cells was measured with the lucigenin assay (20) as described previously (18).

**Measurement of Enzyme Release**—Release of β-glucuronidase and N-acetyl-β-glucosaminidase enzymes from HL-60 cells was assessed as described (21). Briefly, differentiated HL-60 cells (3.5–5 × 10⁵ cells) were suspended in 500 μl of Hanks’ balanced salt solution and incubated for 5 min at 37 °C in the presence of cytochalasin B (5 μg/ml). Reactions initiated by the addition of DMLP were conducted for 5 min at 37 °C and terminated by placement of the tubes on ice. After cell pelleting, activities of β-glucuronidase and N-acetyl-β-glucosaminidase enzymes in supernatant fluids and cell lysates were determined as described (22). Release of the enzymes is given as a percentage of the total cellular content.

**Data Presentation and Analysis**—Unless otherwise stated, results are presented as the mean ± S.E. of three independent experiments, each done in duplicates or triplicates. Statistical analysis was performed by Student’s two-tailed t test for unpaired data.

**RESULTS**

**Formyl Peptide Receptor and G Protein Stimulation of Sphingosine Kinase in HL-60 Cells**—First, we investigated whether the formyl peptide receptor activates the sphingosine kinase in dibutyryl cAMP-differentiated HL-60 cells. For this, formation of [³H]SPP from [³H]sphingosine was determined for various periods of time at 37 °C in the absence and presence of the receptor agonist fMLP, which was applied simultaneously with the radiolabel. Cellular uptake of [³H]sphingosine was fast, reaching 76% of total added after 30 s and 84% after 5 min of incubation, and was not influenced by fMLP (data not shown). Basal conversion of [³H]sphingosine by sphingosine kinase to [³H]SPP was also rapid, and within 2–5 min a plateau of [³H]SPP was reached (Fig. 1A). Activation of the formyl peptide receptor by 10 μM fMLP induced a rapid and transient increase in [³H]SPP production (Fig. 1B). After 2 min, the increase in [³H]SPP reached a maximum of 63 ± 11% (n = 6) above basal [³H]SPP formation in unstimulated cells. Comparable increases in [³H]SPP production were observed in cells stimulated for 2 min with 0.1 μM and 1 μM fMLP. When the measurements were performed in the presence of the sphingosine kinase inhibitors, DHS (30 μM) or DMS (15 μM) (6, 8, 23–25), basal and fMLP-stimulated formation of [³H]SPP were inhibited by 80–90% (data not shown). Together, these results indicate that the formyl peptide receptor is capable of stimulating SPP production by sphingosine kinase in differentiated cells.
To investigate the signaling pathway leading to sphingosine kinase activation, we studied first whether G\textsubscript{i}-type G proteins are involved in the formyl peptide receptor action. Thus, formation of \[^{3}H\]SPP was measured in control and PTX-treated HL-60 cells. Stimulation of control cells for 2 min with 10 \(\mu\)M fMLP increased \[^{3}H\]SPP levels by 67 ± 17\% over unstimulated cells (Fig. 2A). PTX treatment (50 ng/ml, 20 h) had no effect on basal \[^{3}H\]SPP production but completely blocked fMLP-induced stimulation of sphingosine kinase, indicating that activation of sphingosine kinase by the formyl peptide receptor is mediated by G\textsubscript{i}-type G proteins. To directly assess the role of G proteins in sphingosine kinase stimulation, we studied the effects of direct G protein activation on \[^{3}H\]SPP formation in intact cells and in a cell-free system. Direct activation of heterotrimeric G proteins by AlF\textsubscript{4}\(^{2-}\) in intact HL-60 cells led to a rapid and transient production of \[^{3}H\]SPP, in a manner similar to the receptor agonist fMLP (Fig. 2B). \[^{3}H\]SPP levels in cells stimulated for 1, 2, and 5 min with AlF\textsubscript{4}\(^{2-}\) amounted to 117, 142, and 46\%, respectively, above unstimulated control cells.

Sphingosine kinase activity has been found to be present in the cytosol of various cell types (4), and purification of a cytosolic sphingosine kinase from rat kidney has recently been reported (26). Therefore, to study G protein regulation of sphingosine kinase in a cell-free system, the effects of various guanine nucleotides on sphingosine kinase activity were investigated in cytosol prepared from differentiated HL-60 cells. Addition of the stable GTP analog GTP\textsubscript{gS} stimulated SPP production by sphingosine kinase in HL-60 cell cytosol by about 50\% in a concentration-dependent manner, with an EC\textsubscript{50} value of less than 1 \(\mu\)M and a maximal effect at 10–100 \(\mu\)M (Fig. 3A). On the other hand, addition of GTP (100 \(\mu\)M) and the stable GDP analog GDP\textsubscript{bS} (500 \(\mu\)M) did not alter basal sphingosine kinase activity (Fig. 3B). However, GDP\textsubscript{bS} (500 \(\mu\)M) almost fully blocked sphingosine kinase stimulation by GTP\textsubscript{gS} (100 \(\mu\)M). Together, these results indicate that G proteins are signal transduction components in the pathway leading to sphingosine kinase activation in myeloid differentiated HL-60 cells.

**Role of Sphingosine Kinase in Formyl Peptide Receptor Signaling in HL-60 Cells**—Previous studies in other cell types suggested that sphingosine kinase activation represents a Ca\textsuperscript{2+} signaling pathway for some plasma membrane receptors (8, 9, 14). Because fMLP stimulated sphingosine kinase, we investigated the role of this pathway in Ca\textsuperscript{2+} mobilization in HL-60 cells.

---

**Fig. 2.** G protein stimulation of sphingosine kinase in intact HL-60 cells. Formation of \[^{3}H\]SPP from \[^{3}H\]sphingosine was determined for 2 min in control and PTX-pretreated HL-60 cells in the absence (Basal) and presence of 10 \(\mu\)M fMLP (A) or in control HL-60 cells for the indicated periods of time in the absence (Basal) and presence of AlF\textsubscript{4}\(^{2-}\) (10 mM NaF plus 10 \(\mu\)M AlCl\textsubscript{3}) (B). In A, values are expressed as percentages of \[^{3}H\]SPP production relative to unstimulated control cells. *, significantly different from basal SPP formation (p < 0.05).

**Fig. 3.** G protein stimulation of sphingosine kinase activity in cytosol of HL-60 cells. Sphingosine kinase activity was determined in HL-60 cytosolic fractions in the presence of the indicated concentrations of GTP\textsubscript{gS} (A) or in the absence (Basal) and presence of 100 \(\mu\)M GTP\textsubscript{gS}, 500 \(\mu\)M GDP\textsubscript{bS}, GTP\textsubscript{gS} plus GDP\textsubscript{bS}, or 100 \(\mu\)M GTP as described under “Experimental Procedures” (B). Values are expressed as percentages of basal sphingosine kinase activity, amounting to 45 ± 6 pmol SPP/mg protein and 20 min (n = 14). *, significantly different from basal sphingosine kinase activity (p < 0.05).
Effects of sphingosine kinase inhibitors on fMLP-stimulated [Ca$^{2+}$], increases. In the presence of 1 mM extracellular Ca$^{2+}$, increases in [Ca$^{2+}$], induced by fMLP at the indicated concentrations were determined in HL-60 cells pretreated for 1 min without (Control) and with 30 μM DHS (A), or [Ca$^{2+}$], increases induced by 0.1 μM fMLP were determined in cells pretreated for 1 min with DHS or DMS at the indicated concentrations (B). In B, values are given as percentages of control, the 100% value reflecting the [Ca$^{2+}$], increase induced by 0.1 μM fMLP in the absence of inhibitors (519 ± 71 nM, n = 6). C, in the absence of extracellular Ca$^{2+}$, fMLP (1 μM)-induced [Ca$^{2+}$], increase was determined in HL-60 cells pretreated for 1 min without (Control) and with 30 μM DHS. Superimposed tracings are shown. Addition of fMLP is indicated by the arrow.

HL-60 cells. Stimulation of HL-60 cells with fMLP (0.1 nM to 0.1 μM) in the presence of 1 mM extracellular Ca$^{2+}$ markedly increased [Ca$^{2+}$], (Fig. 4A). Maximal increase by about 500 nM was observed at 0.1 μM fMLP. In cells pretreated for 1 min with the sphingosine kinase inhibitor DHS (30 μM), [Ca$^{2+}$], increases were reduced by 80–90% at all fMLP concentrations examined, even at a supramaximally effective fMLP concentration of 10 μM (data not shown). Half-maximal inhibition of fMLP (0.1 μM)-stimulated [Ca$^{2+}$], increase by DHS was observed at 5.9 ± 0.4 μM (Fig. 4B). A similar inhibition was observed with DMS, another sphingosine kinase inhibitor (IC$_{50}$ = 5.2 ± 0.9 μM). As exemplified in Fig. 4C, treatment of HL-60 cells with DHS (30 μM) also markedly inhibited the fMLP (1 μM)-stimulated [Ca$^{2+}$], increase measured in the absence of extracellular Ca$^{2+}$.

DHS and DMS by themselves did not cause an increase in [Ca$^{2+}$], in HL-60 cells. To dismiss the possibility that the sphingosine kinase inhibitors might deplete intracellular Ca$^{2+}$ stores, we studied the effect of DHS (30 μM) on [Ca$^{2+}$], elevations induced by the endoplasmic reticulum Ca$^{2+}$-ATPase inhibitor, thapsigargin, in the absence of external Ca$^{2+}$. In contrast to fMLP stimulation, maximal [Ca$^{2+}$], increases induced by thapsigargin (1 μM) were not affected by DHS (control cells, 73 ± 4 nM; DHS-treated cells, 87 ± 4 nM). The time to peak [Ca$^{2+}$], after thapsigargin addition, however, was significantly (p < 0.05) increased from 60 ± 8 s in control cells to 103 ± 9 s in DHS-treated cells (data not shown). The inhibitory action of DHS and DMS on fMLP-induced [Ca$^{2+}$], increase was not caused by inhibition of PLC stimulation. Preincubation of HL-60 cells for 10 min with 30 μM DHS or 15 μM DMS, strongly reducing Ca$^{2+}$ mobilization, neither altered basal nor inhibited fMLP (10 μM)-stimulated production of either IP$_3$, or total inositol phosphates (Table I). Furthermore, release of 45Ca$^{2+}$ induced by IP$_3$ (0.3–20 μM) from 45Ca$^{2+}$-preloaded saponin-permeabilized cells, measured as described before (27), was not affected by pretreatment of HL-60 cells for 10 min with 30 μM DHS. Finally, because DHS and DMS can act as protein kinase C (PKC) inhibitors (28), we examined whether the inhibition of Ca$^{2+}$ signaling might be due to PKC inhibition. However, in contrast to DHS and DMS, pretreatment of HL-60 cells with the PKC inhibitors staurosporine (100 nM, 3 min), or bisindolylmaleimide I (100 nM, 15 min) had no effect on the fMLP (0.1 μM)-induced [Ca$^{2+}$], elevation, amounting to 605 ± 36 nM in control cells and 637 ± 26 and 591 ± 99 nM in staurosporine- and bisindolylmaleimide I-treated cells, respectively (data not shown).

Finally, the role of sphingosine kinase in two well-characterized functional responses of myeloid differentiated HL-60 cells to formyl peptide receptor activation was examined, namely production of superoxide anions by NADPH oxidase and enzyme exocytosis. Pretreatment of differentiated HL-60 cells for 1 min with 30 μM DHS did not affect the superoxide production induced either by a maximally effective concentration of fMLP (1 μM) or by a half-maximally effective one (10 nM) (Fig. 5). On the other hand, staurosporine (100 nM) strongly inhibited (by 70 ± 5%) the fMLP (1 μM)-stimulated production of superoxide anions (data not shown).

In contrast to superoxide production, formyl peptide receptor-stimulated enzyme release was strongly affected by the sphingosine kinase inhibitors. As illustrated in Fig. 6A, pre-treatment of HL-60 cells for 5 min with 30 μM DHS or 15 μM DMS inhibited, by 45–50%, the fMLP (1 μM)-stimulated release of β-glucuronidase. A similar inhibition was observed when the effect of DHS on fMLP (1 μM)-stimulated release of N-acetyl-β-glucosaminidase was examined (Fig. 6B). Pretreatment with DMS inhibited receptor-stimulated release of this enzyme half-maximally and maximally (about 85% inhibition) at 12 and 20 μM DMS, respectively. Basal enzyme release was not affected by the sphingosine kinase inhibitors. In contrast to DHS and DMS, pretreatment of HL-60 cells with staurosporine (100 nM, 3 min) did not affect fMLP-stimulated β-glucuronidase release (data not shown).

**DISCUSSION**

In the present study, we demonstrate that sphingosine kinase catalyzing formation of SPP from sphingosine is rapidly activated by formyl peptide receptors in myeloid differentiated HL-60 cells, a process apparently mediated by Gi-type G proteins, and that sphingosine kinase activity is also stimulated by direct G protein activation, both in intact cells and in a cell-free system. Furthermore, by using the two sphingosine kinase inhibitors DHS and DMS, evidence is provided suggesting that
The stability of GDP analog, which prevents stimulation was prevented by a stable GDP analog, production induced by 1 M HL-60 cells for 1 min with vehicle (Control) or fMLP-stimulated superoxide production. Following pretreatment of HL-60 cells for 5 min with vehicle (Control) or 30 μM DHS or 15 μM DMS at the indicated concentrations (B), release of β-glucuronidase (A) and N-acetyl-β-glucosaminidase (B) was determined in the absence (Basal) and presence of 1 μM fMLP as described under “Experimental Procedures.”

### Table I

| Treatment | Basal 1 mM | Basal 10 nM |
|-----------|------------|------------|
| None      | 12.2 ± 2.2 | 13.4 ± 0.4 |
| DHS       | 11.5 ± 1.2 | 14.7 ± 0.4 |
| DMS       | 10.5 ± 1.7 | 14.9 ± 2.9 |

### Figure 5

Lack of effect of sphingosine kinase inhibition on fMLP-stimulated superoxide production. Following pretreatment of HL-60 cells for 1 min with vehicle (Control), 30 μM DHS or 15 μM DMS at the indicated concentrations (B), release of β-glucuronidase (A) and N-acetyl-β-glucosaminidase (B) was determined in the absence (Basal) and presence of 1 μM fMLP as described under “Experimental Procedures.”

### Figure 6

Effects of sphingosine kinase inhibitors on fMLP-stimulated enzyme release. Following pretreatment of HL-60 cells for 5 min with vehicle (Control), 30 μM DHS or 15 μM DMS (A), or DMS at the indicated concentrations (B), release of β-glucuronidase (A) and N-acetyl-β-glucosaminidase (B) was determined in the absence (Basal) and presence of 1 μM fMLP as described under “Experimental Procedures.”

In agreement with our previous findings on muscarinic receptors in HEK-293 cells (14), we provide evidence that the sphingosine kinase pathway is involved in the formyl peptide receptor-mediated Ca²⁺ mobilization in HL-60 cells. Short-term pretreatment of the cells with the sphingosine kinase inhibitors DHS and DMS potently reduced the fMLP-induced increases in [Ca²⁺], with IC₅₀ values (5–6 μM) in the range of those described for inhibition of sphingosine kinase activity (6, 8, 23–25). Control experiments excluded that suppression of Ca²⁺ signaling by DHS and DMS was caused by PKC inhibition, depletion of internal Ca²⁺ stores, and perturbation of the PLC/IP₃ pathway. It is highly unlikely that the intracellularly formed SPP is released from the cells and stimulates the cells in an autocrine manner, i.e. by activation of plasma membrane sphingolipid receptors. First, there was no evidence of fMLP-stimulated release of [³H]SPP into the medium. Second, similar to results reported before in promyelocytic HL-60 cells (18), extracellularly added SPP only marginally increased [Ca²⁺], in differentiated HL-60 cells (by ≤30 nm at 1 μM SPP). Finally, desensitization of this small SPP-induced Ca²⁺ increase by 70% following overnight treatment of the cells with 1 μM SPP did not affect the formyl peptide receptor-induced [Ca²⁺] increase (data not shown). The lag time between peak [³H]SPP formation and [Ca²⁺] increase most likely results from the time span required for extracellularly applied [³H]sphingosine to cross the plasma membrane and reach intracellular sphingo-
Exocytosis, the activated receptor generates additional signals required for mobilization, which may explain the delay in Ca$^{2+}$ release induced by thapsigargin, without reducing the maximal [Ca$^{2+}$]$_i$ increase by this agent. In lacrimal acinar cells, the thapsigargin-induced [Ca$^{2+}$]$_i$ rise due to leakage of Ca$^{2+}$ from IP$_3$-sensitive stores has been shown to be dependent on basal levels of IP$_3$ (29). Thus, the absence of SPP after DHS treatment may explain the delay in Ca$^{2+}$ release induced by thapsigargin. Our data thus suggest that although the formyl peptide receptor strongly stimulates PLC-catalyzed IP$_3$ formation, there is a tight correlation between [Ca$^{2+}$]$_i$ and enzyme release, whereas the PKC inhibitor induced by fMLP, both at maximally and half-maximally effective concentrations of this stimulus, whereas the PKC inhibitor strongly stimulates NADPH oxidase in phagocytic cells involves several membrane mechanisms, including increase in [Ca$^{2+}$]$_i$ and PKC activation but also by Ca$^{2+}$- and PKC-independent pathways (31–33). Treatment of HL-60 cells with the sphingosine kinase inhibitor, DHS (30 µM), causing marked inhibition of fMLP-induced [Ca$^{2+}$]$_i$ increase, had no effect on superoxide production induced by fMLP, both at maximally and half-maximally effective concentrations of this stimulus, whereas the PKC inhibitor stauroporine strongly inhibited the fMLP response. These results indicate that sphingosine kinase is apparently not involved in the signaling pathway to NADPH oxidase. In contrast to superoxide production, there is a tight correlation between rises in [Ca$^{2+}$]$_i$ and enzyme exocytosis in HL-60 granulocytes (34). Pretreatment of differentiated HL-60 cells with DHS and DMS strongly inhibited the fMLP-stimulated release of β-glucuronidase and N-acyetyl-β-glucosaminidase. Moreover, the inhibition of fMLP-induced N-acyetyl-β-glucosaminidase release and [Ca$^{2+}$]$_i$ increase by DMS exhibited a similar concentration dependence, strongly suggesting that inhibition of enzyme release is, at least to a major extent, due to blockade of Ca$^{2+}$ mobilization. Because fMLP-stimulated enzyme release was not completely inhibited by DHS or DMS at concentrations effectively blocking fMLP-induced [Ca$^{2+}$]$_i$, rises, it appears that the activated receptor generates additional signals required for exocytosis, e.g. stimulation of phosphatidylinositol 3-kinase.

Finally, the differential sensitivity of the cellular events triggered by the formyl peptide receptor to DHS and DMS (i.e. no effect on PLC stimulation and superoxide production, but marked inhibition of Ca$^{2+}$ mobilization and enzyme release) underlines the specificity of the sphingosine kinase inhibitors.

In conclusion, this study demonstrates for the first time that formyl peptide receptors stimulate through PTX-sensitive G$i$-type G proteins SPP production by sphingosine kinase in myeloid differentiated HL-60 cells and that this enzyme is also stimulated by direct G protein activation. Furthermore, evidence is provided that the sphingosine kinase/SPP pathway is involved in Ca$^{2+}$ and exocytosis signaling pathways but apparently not in superoxide production in phagocytic cells.

REFERENCES

1. Hannun, Y. A. (1994) J. Biol. Chem. 269, 3125–3128
2. Heller, R. A., and Kro¨nke, M. (1994) J. Membr. Biol. 136, 5–9
3. Kolesnik, R., and Golde, D. W. (1994) Cell 77, 325–328
4. Spiegel, S., and Milstien, S. (1995) J. Membr. Biol. 144, 225–237
5. Meyer zu Heringdorf, D., van Koppen, C. J., and Jakobs, K. H. (1997) FEBS Lett. 410, 34–38
6. Olivera, A., and Spiegel, S. (1993) Nature 365, 557–560
7. Bornfeldt, K. E., Graves, L. M., Raines, E. W., Igarashi, Y., Wayman, G., Yamamura, S., Yatomi, Y., Sidhu, J. S., Krebs, E. G., Hakomori, S., and Ross, R. (1995) J. Cell Biol. 130, 193–206
8. Choi, O. H., Kim, J.-H., and Kinet, J.-P. (1996) Nature 380, 634–636
9. Melendez, A., Plito, R. A., Gilloly, D. J., Harnett, M. M., and Allen, J. M. (1990) J. Biol. Chem. 273, 3939–4942
10. Qiao, L., Kuzikowski, A. P., Olivera, A., and Spiegel, S. (1996) Bioorg. Med. Chem. Lett. 8, 711–714
11. Ghosh, T. K., Bhan, J., and Gill, D. L. (1990) Science 248, 1653–1656
12. Ghosh, T. K., Bhan, J., and Gill, D. L. (1994) J. Biol. Chem. 269, 23628–23635
13. Mattie, M., Brooker, G., and Spiegel, S. (1994) J. Biol. Chem. 269, 3181–3188
14. Meyer zu Heringdorf, D., Lass, H., Alemay, R., Laser, K. T., Neumann, E., Zhang, C., Schmidt, M., Rauzen, U., Jakobs, K. H., and van Koppen, C. (1998) EMBO J. 17, 2839–2847
15. Ye, R. D., and Boulay, F. (1997) Adv. Pharmacol. 39, 221–298
16. Gierschik, P., Sidipoulos, D., and Jakobs, K. H. (1989) J. Biol. Chem. 264, 21470–21476
17. Wieland, T., Liedel, K., Kaldenberg-Stasch, S., Meyer zu Heringdorf, D., Schmidt, M., and Jakobs, K. H. (1995) Naunyn-Schmiedeberg’s Arch. Pharmacol. 351, 329–336
18. van Koppen, C. J., Meyer zu Heringdorf, D., Zhang, C., Laser, K. T., and Jakobs, K. H. (1998) Mol. Pharmacol. 49, 965–961
19. Schmidt, M., Biniek, C., van Koppen, C. J., Michel, M. C., and Jakobs, K. H. (1995) Naunyn-Schmiedeberg’s Arch. Pharmacol. 352, 469–476
20. Bhakdi, S., and Martin, E. (1991) Infect. Immun. 59, 2955–2962
21. Wenzel-Seifert, K., and Seifert, R. (1999) Immunobiology 181, 298–316
22. Abadom, D. R. (1986) Methods Enzymol. 132, 95–180
23. Buehrer, B. M., and Bell, R. M. (1992) J. Biol. Chem. 267, 3135–3149
24. Olivera, A., Zhang, H., Carlson, R. O., Mattie, M. E., Schmidt, R. R., and Spiegel, S. (1994) J. Biol. Chem. 269, 17924–17930
25. Yatomi, J., Ruan, F., Megdijch, T., Toyokuni, T., Hakomori, S., and Igarashi, Y. (1996) Biochemistry 35, 626–633
26. Olivera, A., Kehama, T., Tu, Z., Milstien, S., and Spiegel, S. (1998) J. Biol. Chem. 273, 12076–12083
27. Meyer zu Heringdorf, D., Niederdrang, N., Neumann, E., Frode, R., Lass, H., Koppen, C. J., and Jakobs, K. H. (1998) Eur. J. Pharmacol. 354, 113–122
28. Igarashi, Y., Hakomori, S., Toyo Kuni, T., Dean, B., Fujita, S., Sugimoto, M., Ogawa, T., El-Ghendy, K., and Racker, E. (1989) Biochem. Biophys. Res. Commun. 165, 113–122
29. Smith, P. M., and Gallacher, D. (1994) Biochem. J. 299, 37–40
30. Mathias, D. S., Nikolaiwa, K., Michiakawa, T., Miyawaki A., and Ives, H. E. (1998) Am. J. Physiol. 274, C1456–C1465
31. Morel, F., Dousiere, J., and Vignais, P. V. (1991) Eur. J. Biochem. 201, 523–546
32. Segal, A. W., and Abe, A. (1993) Trends Biochem. Sci. 18, 164–168
33. Ridley, A. J. (1997) Curr. Biol. 7, 710–712
34. Nüe, O., Serrander, L., Foyouzi-Youssef, R., Monod, A., Lew, D. P., and Krause, K.-H. (1997) J. Biol. Chem. 272, 28360–28367
Formyl Peptide Receptor Signaling in HL-60 Cells through Sphingosine Kinase
Regina Alemany, Dagmar Meyer zu Heringdorf, Chris J. van Koppen and Karl H. Jakobs

J. Biol. Chem. 1999, 274:3994-3999.
doi: 10.1074/jbc.274.7.3994

Access the most updated version of this article at http://www.jbc.org/content/274/7/3994

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 34 references, 16 of which can be accessed free at http://www.jbc.org/content/274/7/3994.full.html#ref-list-1