Sphingosine-1-phosphate (SPP) has attracted much attention as a possible second messenger controlling cell proliferation and motility and as an intracellular Ca\textsuperscript{2+}-releasing agent. Here, we present evidence that SPP activates a G protein-coupled receptor in the plasma membrane of various cells, leading to an increase in cytoplasmic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]i), inhibition of adenyl cyclase, and opening of G protein-regulated potassium channels. In human embryonic kidney (HEK) cells, SPP potently (EC\textsubscript{50}, 2 nM) and rapidly increased [Ca\textsuperscript{2+}]i in a pertussis toxin-sensitive manner. Pertussis toxin-sensitive increase in [Ca\textsuperscript{2+}]i was also observed with sphingosylphosphorylcholine (EC\textsubscript{50}, 460 nM), whereas other sphingolipids, including ceramide-1-phosphate, N-palmitoyl-sphingosine, psychosine, and α-erythro-sphingosine at micromolar concentrations did not or only marginally increased [Ca\textsuperscript{2+}]i. Furthermore, SPP inhibited forskolin-stimulated cAMP accumulation in HEK cells and increased binding of guanosine 5′-3-O-(thio)triphosphate to HEK cell membranes. Rapid [Ca\textsuperscript{2+}]i responses were also observed in human transitional bladder carcinoma (J82) cells, monkey COS-1 cells, mouse NIH 3T3 cells, Chinese hamster ovary (CHO-K1) cells, and rat C6 glioma cells, whereas human HL-60 leukemia cells and human erythroleukemia cells failed to respond to SPP. In guinea pig atrial myocytes, SPP activated G\textsubscript{i}, protein-regulated inwardly rectifying potassium channels. Activation of these channels occurred strictly when SPP was applied at the extracellular face of atrial myocyte plasma membrane as measured in cell-attached and inside-out patch clamp current recordings. We conclude that SPP, in addition to its proposed direct action on intracellular Ca\textsuperscript{2+} stores, interacts with a high affinity G\textsubscript{i}, protein-coupled receptor in the plasma membrane of apparently many different cell types.

Over the past years, sphingolipids have emerged as important second messengers of cellular signaling (1–3). Following stimulation of cells with nerve growth factor, tumor necrosis factor-α, and interleukin-1β, sphingomyelinases are activated leading to the generation of ceramide, which can be further metabolized to sphingosine and sphingosine-1-phosphate (SPP)\textsuperscript{1} by the action of ceramidase and sphingosine kinase, respectively. Although ceramide and sphingosine have been the subject of extensive studies, recently, attention has also been focused on SPP. This sphingolipid has been demonstrated to be involved in a multitude of processes. Activation of sphingosine kinase and enhanced formation of SPP was shown to be induced by platelet-derived growth factor, with SPP being implicated as an important second messenger for the promotion of DNA synthesis in Swiss 3T3 fibroblasts (4). DNA synthesis and cell division of 3T3 cells could also be noted when SPP was added exogenously to intact 3T3 cells (5). At low concentrations, SPP is also able to inhibit efficiently motility and invasiveness of various tumor cells that cannot be mimicked by sphingosine or N-methylated sphingosines (6). Another important action of SPP has emerged from the observation in various cellular systems that SPP can cause release of Ca\textsuperscript{2+} from internal stores by a non-inositol 1,4,5-trisphosphate receptor-mediated mechanism (7–9). On the other hand, a rapid increase in cytoplasmic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]i) could also be obtained by direct application of SPP to intact Swiss 3T3 cells (7).

Thus, SPP appears to be an important component in the signaling system that is involved in Ca\textsuperscript{2+} release and in the regulation of cell growth and motility. However, thus far, the molecular targets of SPP have been rather elusive. One possible molecular target may be an intracellular Ca\textsuperscript{2+}-permeable channel located in the endoplasmic reticulum, which is gated by SPP (7–9). This channel may be reached by exogenously applied SPP. A unique feature of SPP response on intact cells, however, is the immediate and transient rise in [Ca\textsuperscript{2+}]i (7). We therefore studied whether SPP may also activate a plasma membrane receptor rather than surpassing the plasma membrane to activate a putative intracellular Ca\textsuperscript{2+}-permeable channel in the endoplasmic reticulum. In the present report, we provide evidence for this hypothesis and demonstrate that SPP at nanomolar concentrations activates pertussis toxin (PTX)-sensitive guanine nucleotide-binding proteins (G proteins) via a plasma membrane receptor apparently present in many cell types.

**EXPERIMENTAL PROCEDURES**

Materials—Psychosine, sphingosylphosphorylcholine, lysophosphatidic acid (LPA), and bovine serum albumin (lot. 62H0154) were from Sigma. SPP and N-palmitoyl-sphingosine were obtained from Biomol.\textsuperscript{2}

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\textsuperscript{3}The abbreviations used are: SPP, sphingosine-1-phosphate; PTX, pertussis toxin; G protein, guanine nucleotide-binding protein; LPA, lysophosphatidic acid; GTP\textsubscript{y5}, guanosine 5′-3-O-(thio)triphosphate; HEK, human embryonic kidney.
c-erythro-Sphingosine and C₂-ceramide-1-phosphate were purchased from Calbiochem. Fura-2/AM was from Molecular Probes, PTX was from List Laboratories, and [³⁵S]GTP•S (1322 Ci/mmol) was from DuPont NEN. All other reagents were from previously described sources (10–14). Stock solutions of SPP (1 mM) were made up in 100% methanol and stored at −20 °C. Prior to the experiments, SPP solutions were dried down, and SPP was dissolved in phosphate-buffered saline as a complex with bovine serum albumin (4 mg/ml) to a final concentration of 1 mM. c-erythro-Sphingosine was dissolved in absolute ethanol (100 mM) and diluted to 1 mM in 4 mg/ml bovine serum albumin. Sphingosylphosphorylcholine was dissolved as a complex in phosphate-buffered saline (1 mM) with bovine serum albumin (4 mg/ml). Dilutions of these stock solutions were in phosphate-buffered saline with bovine serum albumin and stored at −20 °C. Prior to the experiments, SPP solutions were dried down, and SPP was dissolved in phosphate-buffered saline as a complex with bovine serum albumin (4 mg/ml) to a final concentration of 1 mM. SPP was dissolved in phosphate-buffered saline with bovine serum albumin (4 mg/ml). Dilutions of these stock solutions were in phosphate-buffered saline with 1 mg/ml bovine serum albumin. Bovine serum albumin by itself had no effect on [Ca²⁺]i.

Cell Culture and PTX Treatment—Control human embryonic kidney (HEK) 293 cells and HEK 293 cells stably expressing the human m3 muscarinic acetylcholine receptor subtype were cultured as described in detail previously (10, 11). In some experiments, cells were treated for 16 h with 100 ng/ml PTX to test the role of PTX-sensitive G proteins in SPP signaling. Atrial myocytes from hearts of adult guinea pigs were isolated and cultured in bicarbonate-buffered M199 medium as described previously (12). Atrial cells were plated at a low density (several hundred cells per 35-mm culture dish) and used experimentally for up to 8 days.

GTP•S Binding Assay—Binding of GTP•S to HEK 293 cell membranes prepared as described before (10) was performed in a reaction mixture (100 μl) containing 0.4 mM [³⁵S]GTP•S, 5 mM MgCl₂, 1 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol, 1 μM GTP, 50 mM triethanolamine-HCl, pH 7.4, and the indicated additions. The reaction started by the addition of membranes (about 5–30 μg of protein/tube) was conducted for 30 min at 30 °C. Separation of membrane-bound and free radioactivity and determination of nonspecific binding were performed as described before (10).

Measurement of cAMP Accumulation—HEK 293 cells grown to 90–100% confluency on 35-mm plates were preincubated for 20 min at 37 °C with 5 mM theophylline to inhibit phosphodiesterases. Then, cells were stimulated for 5 min with 50 μM forskolin plus 10 μM SPP or vehicle. The reaction was stopped, and cAMP was measured as described previously (13). cAMP was measured by the Lowry method. [Ca²⁺]i Measurements—[Ca²⁺]i was determined with the fluorescent calcium indicator dye Fura-2 in a Perkin Elmer LS-5B spectrofluorimeter equipped with a fast-filter device as described before (14). Briefly, cells resuspended at approximately 0.5–1.0 × 10⁶ cells/ml were incubated with 1 μM Fura-2/AM for 1 h at 37 °C in a buffer containing 137 mM NaCl, 2.7 mM KCl, 0.9 mM CaCl₂, 0.5 mM MgCl₂, 6.5 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 1 mg/ml bovine serum albumin, and 1 mg/ml d-glucose at pH 7.4. Thereafter, cells were washed twice, resuspended in fresh buffer, and used for fluorescence measurements within the next hour. To remove dyes having leaked into the medium, aliquots were pelleted in a microcentrifuge, resuspended in fresh prewarmed medium without bovine serum albumin, and immediately transferred to a thermostatted cuvette (37 °C) in the spectrofluorimeter. Excitation was alternating at 340 and 380 nm with emission being read at 495 nm. Fluorescence data were converted into Ca²⁺ concentration with software supplied by the manufacturer. In some experiments, extracellular Ca²⁺ was chelated by addition of 5 mM EGTA 30 s prior to agonist exposure (corresponding to 17 mM free Ca²⁺).

Measurement of Iₖ(ACh) Channels in Atrial Myocytes—Activity of inwardly rectifying K⁺ channels (Iₖ(ACh)) in guinea pig atrial myocytes was measured using the cell-attached and the inside-out configuration of the patch clamp technique (15) as described in detail previously (12, 16). In brief, pipettes fabricated from borosilicate glass with filament (Clark, Pangbourne, UK) were filled with a solution containing 150 mM KCl, 2 mM CaCl₂, and 10 mM HEPES/KOH, pH 7.4. DC resistance of the pipettes was 2–4 megaohm. Current measurement was performed by means of a patch clamp amplifier (LM/EPC-7, List, Darmstadt, Germany).
was replaced by HEPES-buffered 150 mM KCl solution supplemented predominantly increases [Ca\textsuperscript{2+}]. Following the chelation of extracellular Ca\textsuperscript{2+}, the [Ca\textsuperscript{2+}]i curve was rather shallow, with a calculated Hill coefficient of 0.44 ± 0.05. Out of the other sphingolipids studied, ceramide-1-phosphate, psychosine, and N-palmitoyl-sphingosine at 10 μM did not alter [Ca\textsuperscript{2+}], (data not shown), and ω-erythro-sphingosine at 10 μM only marginally elevated [Ca\textsuperscript{2+}]. In contrast, sphingosylphosphorylcholine increased [Ca\textsuperscript{2+}]i in HEK 293 cells to the same maximal level as SPP. Responses to sphingosylphosphorylcholine were as fast as those to SPP but were found at much higher concentrations (EC50, 457 ± 31 nM). Similar to the SPP response, [Ca\textsuperscript{2+}]i increases induced by sphingosylphosphorylcholine were largely blunted by PTX pretreatment (data not shown). LPA shares some structural similarity to SPP, i.e., a long hydrocarbon chain with a terminal phosphate group, and is known to exert many SPP-like effects (17). Therefore, we tested whether SPP acts by activating LPA receptors. For this, we investigated whether [Ca\textsuperscript{2+}]i transients induced by LPA were affected by a prior challenge with SPP. As shown in Fig. 3, this was not the case. Yet, the [Ca\textsuperscript{2+}]i response to SPP (1 μM) was abolished following a first SPP challenge. In the reverse order of addition, the SPP response was not affected following a challenge with LPA (1 μM), while prior activation with LPA abolished the effect of a second LPA challenge. From these results, it can be concluded that SPP does not increase [Ca\textsuperscript{2+}]i by activating the LPA receptor.

Since the [Ca\textsuperscript{2+}]i-elevating action of SPP was largely reduced by PTX treatment, we studied whether SPP by activating PTX-sensitive G proteins inhibits adenyl cyclase. As shown in Fig. 4, SPP (10 μM) decreased forskolin-stimulated cAMP accumulation in HEK 293 cells by 30–40%. This inhibitory response was completely blocked by pretreating cells with PTX (100 ng/ml, 16 h).

Finally, we determined whether SPP activates G proteins, by measuring binding of the labeled stable GTP analog GTPγS to membranes of HEK 293 cells (Fig. 5). SPP (1 μM) increased binding of [35S]GTPγS to membranes of HEK 293 cells by about 50%. An increase of similar magnitude (about 90%) was observed in response to carbachol (1 mM), which activates the m3 muscarinic acetylcholine receptor stably expressed in the same HEK 293 cells as described before (10). This HEK 293 cell clone showed identical [Ca\textsuperscript{2+}]i responses to SPP as untransfected HEK 293 cells.

SPP-induced [Ca\textsuperscript{2+}]i responses are cell type-specific—The data thus far presented suggested that SPP activates in HEK 293 cells a plasma membrane receptor coupled to PTX-sensitive G proteins, although a direct G protein activation could not be excluded. To corroborate the receptor hypothesis, we therefore set out to test a variety of other cell types for their ability to generate a rapid, transient increase in [Ca\textsuperscript{2+}]i in response to SPP. In addition to HEK 293 cells, SPP (10 μM) increased [Ca\textsuperscript{2+}]i in very divergent cell types, such as human transitional bladder carcinoma (J82) cells, monkey COS-1 cells, mouse NIH 3T3 cells, Chinese hamster ovary (CHO-K1) cells, and rat C6 glioma cells (Table I). Notably, however, differentiated human leukemia (HL–60) cells and human erythroleukemia cells failed to respond to 10 μM SPP but were otherwise strongly responsive to known receptor agonists, e.g., N-formyl-methionyl-leucyl-phenylalanine and thrombin (data not shown).

To study whether the PTX sensitivity of the [Ca\textsuperscript{2+}]i response was cell type-dependent, we chose to test in NIH 3T3 fibroblasts whether PTX-sensitive G proteins are involved in SPP-induced [Ca\textsuperscript{2+}]i increases as well. While in untreated NIH 3T3 cells SPP (10 μM) elevated [Ca\textsuperscript{2+}]i by 130 nM, in PTX-pretreated NIH 3T3 cells SPP-induced [Ca\textsuperscript{2+}]i increase was completely abolished (data not shown).

SPP activates I\textsubscript{K(ACh)} in atrial myocytes—After finding activation of PTX-sensitive cellular responses, we tested whether SPP activates muscarinic acetylcholine receptor-regulated, inwardly rectifying potassium channels (I\textsubscript{K(ACh)}) in atrial myo...
cytes. Receptor regulation of these channels is mediated by PTX-sensitive G proteins (18–20). SPP is a potent and efficient activator of $I_{K(ACh)}$ in guinea pig atrial myocytes, as studied in whole cell recording configurations, and its effect is fully PTX-sensitive (21). This experimental system was, therefore, used to address the question of whether the action of SPP is brought about strictly from the extracellular face of the plasma membrane, where the interaction of SPP with its putative receptor should take place. In the cell-attached mode, a membrane channel under the mouth of the pipette is accessible to signals from outside this area only if these can transverse the plasma membrane (15, 16). Thus, if SPP were producing its effect by direct interaction with G proteins, it should cause activation of $I_{K(ACh)}$ channels if applied to the cell outside the membrane patch isolated by the pipette, whereas a strictly extracellular action should fail to produce activation of $I_{K(ACh)}$ under the pipette. As illustrated in Fig. 6, which shows a slow speed recording of basal $I_{K(ACh)}$ activity, superfusion of the cells with 1 $\mu$M SPP failed to produce any change in channel activity, whereas the addition of 1 $\mu$M SPP to the pipette solution did lead to a significant increase in $I_{K(ACh)}$ activity.

**Fig. 3.** Lack of cross-desensitization of SPP- and LPA-induced $[Ca^{2+}]_i$ increases in HEK 293 cells. $[Ca^{2+}]_i$ increases in HEK 293 cells were determined upon addition of either 1 $\mu$M SPP or 1 $\mu$M LPA as indicated. Addition of SPP or LPA is indicated by the arrows.

**Fig. 4.** SPP-induced inhibition of forskolin-stimulated cAMP accumulation in HEK 293 cells. cAMP levels were determined in HEK 293 cells pretreated without and with PTX (100 ng/ml, 16 h) in the presence of 50 $\mu$M forskolin (Fors) or forskolin plus 10 $\mu$M SPP as described under “Experimental Procedures.” Data are from one set of experiments (each done in triplicate), representative of two independent experiments that yielded similar results.
TABLE I

Effect of SPP on [Ca\(^{2+}\)]\(_i\) in various cell lines

| Cell line                             | Peak change in [Ca\(^{2+}\)]\(_i\) (nM) |
|---------------------------------------|------------------------------------------|
| Responders                            |                                          |
| Human embryonic kidney (HEK 293) cells | 299 ± 15                                 |
| Human transitional bladder carcinoma (l 82) cells | 235 ± 15                                 |
| Monkey COS-1 cells                    | 138 ± 34                                 |
| Mouse NIH 3T3 fibroblasts             | 95 ± 14                                  |
| Chinese hamster ovary (CHO-K1) cells  | 92 ± 16                                  |
| Rat C6 glioma cells                   | 60 ± 10                                  |
| Non-responders                        |                                          |
| Human leukemia HL-60 cells             | <25                                      |
| Human erythroleukaemia cells           | <25                                      |

Fig. 6. Lack of an effect of SPP on \(I_{\text{K(ACh)}}\) Channel activity in atrial myocyte-attached membrane patch. Channel current was measured using an approximately symmetrical K\(^+\) distribution across the patch, whereas the bath solution contained 200 mM K\(^+\) to “damp” the resting potential (\(E_R\)) at around −50 mV. Membrane potential across the patch was \(E_R\) − 60 mV, resulting in a unitary inward current through open K\(^+\) channels of approximately 4 pA. This experimental condition was used because of the strongly inward-rectifying properties of \(I_{\text{K(ACh)}}\) channels, which pass hardly any current in the outward direction.

Further evidence for a receptor-mediated action of SPP was obtained from single channel recordings in the inside-outside mode. When the pipette solution, facing the original outer face of the plasma membrane, was supplemented with 1 μM SPP, exposure of the inside of the membrane to GTP (50 μM) resulted in a dramatic increase in channel activity (Fig. 7, trace A). In the absence of SPP, exposure to GTP alone caused a slight increase in channel activity (trace B), which is due to the fact that also basal activity of this channel is G protein-regulated (22). Notably, the same low degree of channel activity was obtained when GTP (50 μM) and SPP (1 μM) were applied simultaneously to the intracellular face of the isolated patch (trace C). Peak values (\(n = 4\)) for \(nP_0\) upon exposure of the patches to GTP were 4.6 ± 3.5 (A), 0.31 ± 0.15 (B), and 0.26 ± 0.19 (C). In summary, the patch clamp data clearly demonstrate that SPP acts only from the extracellular face of the plasma membrane and requires GTP for activation of \(I_{\text{K(ACh)}}\) channels.

**DISCUSSION**

In the present study, we tested the hypothesis that exogenous SPP acts on intact cells via activation of a plasma membrane receptor. We demonstrate that SPP rapidly increases [Ca\(^{2+}\)]\(_i\) in various cells, inhibits forskolin-stimulated adenyl cyclase in HEK 293 cells, and activates \(I_{\text{K(ACh)}}\) channels in atrial myocytes. These responses were either to a large extent a direct interaction with G proteins reached after transversing the plasma membrane.

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During the course of this study, the groups of Spiegel and Sturgill (23, 24) reported that exogenous SPP decreases cellular cAMP levels, stimulates inositol phosphate formation, increases [Ca\(^{2+}\)], activates mitogen-activated protein kinase, and stimulates DNA synthesis in Swiss 3T3 fibroblasts and that these cellular actions of SPP are largely or completely abolished by PTX pretreatment. These observations led the authors to conclude that SPP may selectively activate PTX-sensitive G proteins in a receptor-independent fashion or alternatively activate a specific cell surface receptor that is coupled to these G proteins. Although the data reported in Swiss 3T3 cells are, at least partially, in agreement with those reported herein, there are also important differences. First, the effects of

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SPP in Swiss 3T3 cells were observed at rather high concentrations (EC50 values of about 2 μM) compared to about 3 orders of magnitude lower concentrations of SPP (EC50 values of about 2 nM) required to increase [Ca2+]i in HEK 293 cells and to activate IK(ACh) channels in atrial myocytes (21). Second and most important, these reports left open the crucial question about the site of action of exogenous SPP.

Several observations suggest that SPP binds to a specific SPP receptor in the plasma membrane. SPP was effective in the nanomolar range (EC50 of 2 nM), whereas various other sphingolipids, including ceramide-1-phosphate, o-erythro-sphingosine, psychosine, and N-palmitoyl-sphingosine, did not or only marginally increase [Ca2+]i in HEK 293 cells, even at micromolar concentrations. Likewise, the structurally related phospholipid LPA, which effectively elevated [Ca2+]i at nanomolar concentrations, did not desensitize the SPP-induced [Ca2+]i response in HEK 293 cells at micromolar concentrations. This lack of cross-desensitization for induced [Ca2+]i transients in HEK 293 cells is in agreement with a similar study in A431 cells described recently (25). On the other hand, sphingosylphosphorylcholine fully mimicked the SPP response, although about 200-fold higher concentrations of sphingosylphosphorylcholine than of SPP were required for increasing [Ca2+]i in HEK 293 cells. This sphingolipid also mimics SPP’s actions on Ca2+ release from endoplasmic reticulum, however apparently at similar concentrations as of SPP (8, 9). The shallow concentration response curve of SPP compared to the rather steep one of sphingosylphosphorylcholine for increasing [Ca2+]i in HEK 293 cells may even suggest that distinct types of receptors are involved in SPP’s actions.

The synthesis of SPP from sphingosine is catalyzed by the enzyme sphingosine kinase. However, present information about this enzyme is rather scarce. Sphingosine kinase appears to be a cytosolic enzyme in platelets while being membrane-associated in rat brain and other tissues (26). Since our study indicates that exogenous SPP acts at a plasma membrane-located receptor, it will be of interest to study how SPP synthesis is regulated by hormones and growth factors, how SPP is released from cells, and what physiological functions SPP plays in different tissues.

In conclusion, this study demonstrates that SPP can regulate intracellular second messengers and membrane channels through activation of specific receptors apparently present in many different cell types and coupled to PTX-sensitive G-type G proteins. SPP has also been implicated as an intracellular second messenger releasing Ca2+ from internal stores. Thus, the present study suggests that SPP has at least two molecular targets of action, i.e., the proposed sphingolipid-gated Ca2+-permeable channel in the endoplasmic reticulum as well as a high affinity G protein–coupled receptor in the plasma membrane.

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