Differential Pharmacological Properties and Signal Transduction of the Sphingosine 1-Phosphate Receptors EDG-1, EDG-3, and EDG-5*

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Sphingosine 1-phosphate (SPP) is a potent lipid mediator released upon cellular activation. In this report, pharmacological properties of the three G-protein-coupled receptors (GPCRs) for SPP, EDG-1, -3, and -5 are characterized using a Xenopus oocyte expression system, which lacks endogenous SPP receptors. Microinjection of the EDG-3 and EDG-5 but not EDG-1 mRNA conferred SPP-responsive intracellular calcium transients; however, the EDG-5 response was quantitatively much less. Co-expression of EDG-1 receptor with the chimeric Gαq protein conferred SPP responsiveness. Gαq or Gαiα co-injection also potentiated the EDG-5 and EDG-3 mediated responses to SPP. These data suggest that SPP receptors couple differentially to the Gαq and Gαi pathway. All three GPCRs were also activated by sphingosylphosphorylcholine, albeit at higher concentrations. None of the other related sphingolipids tested stimulated or blocked SPP-induced calcium responses. However, suramin, a polyvalent anionic compound, selectively antagonized SPP-activated calcium transients in EDG-3 expressing oocytes with an IC50 of 22 μM, suggesting that it is an antagonist selective for the EDG-3 GPCR isotype. We conclude that the three SPP receptors signal differentially by coupling to different G-proteins. Furthermore, because only EDG-3 was antagonized by suramin, variations in receptor structure may determine differences in antagonist selectivity. This property may be exploited to synthesize receptor subtype-specific antagonists.

Cellular activation results in the remodeling of membrane phospholipids, namely, phosphoglycerolipids and phosphosphingolipids, resulting in the production of polar, bioactive lipid mediators (1). Complex enzymatic pathways are involved in post-receptor activation and release of such mediators, lysosphosphatidic acid (LPA)1 and sphingosine 1-phosphate (SPP) (1, 2). For example, hydrolysis of sphingomyelin, followed by the sequential action of the enzymes ceramidase and sphingosine kinase results in the formation of SPP (3). Although it is not clear how SPP is exported out of the cells, at least in platelets, activation by prothrombotic stimuli results in the formation and export of SPP, achieving high concentrations of SPP in the serum, estimated to be approximately 0.5 μM (4). SPP mediates a number of biological responses, primarily determined in various in vitro systems. For example, SPP induces increases in intracellular calcium (5), stimulates fibroblast proliferation (6), inhibits cellular apoptosis (7), inhibits cell migration (8), induces stress fiber formation (9), regulates adhesion molecule expression (10), and regulates morphogenetic differentiation (10), among others.

Although there is agreement with regard to the broad-spectrum biological actions of SPP (1–3, 11), controversy exists regarding its mode of action (12, 13). Specifically, it is not clear whether various actions of SPP are due to its role as an extracellular mediator that signals via plasma membrane receptors or whether it acts intracellularly as a second messenger molecule. However, these possibilities need not be mutually exclusive. It is nevertheless important to define specific biological responses regulated by SPP as an extracellular mediator and those regulated by intracellular action.

Recently, the G-protein-coupled receptor (GPCR) EDG-1 was identified as a plasma membrane receptor for SPP (10). Specifically, SPP bound to EDG-1 with a Kd of ~ 8 nM, stimulated Gq-dependent extracellular signal-regulated kinase activity, induced the small GTPase Rho-dependent adherens junction assembly and increases in P-cadherin levels (10). Two independent groups also concluded that EDG-1 is a Gq-coupled receptor for SPP (14, 15). EDG-1 was originally cloned as an endothelial differentiation gene from phorbol myristic acetate-treated differentiating human endothelial cells (16). These data suggest that platelet-derived SPP, which is secreted during thrombosis, could regulate endothelial cell signaling events via the EDG-1 receptor. EDG-1 is a prototype of a subfamily of GPCRs whose known members include EDG-2/VZG-1 (17), EDG-3 (18), EDG-4 (19), EDG-5/H218/AGR16 (20), and EDG-6 (21). Chun and colleagues (17) showed that EDG-2 (which they termed as VZG-1) is a high affinity receptor for LPA, and regulated Gq- and Gi-dependent events (22). A highly related receptor, EDG-4 was cloned and was shown to be an LPA receptor, which potently stimulated the Gq/PLCβ/calcium signaling pathway (19). EDG-3 and EDG-5, which are closer in sequence identity to EDG-1, responded to low concentrations of SPP in a Xenopus oocyte-based calcium efflux assay and serum response factor-based transcriptional activation assay in Jurkat T-cells (19). These data suggest that EDG-3 and EDG-5, like EDG-1 are high affinity SPP receptors and that EDG-2 and EDG-4 are high affinity LPA receptors. The response of EDG-6 to LPA and SPP is not known.

The three known receptors for SPP, EDG-1, -3, and -5 exhibit overlapping as well as distinct patterns of expression in various tissues (16, 20, 24–26). Although expression studies have by
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and large focused on low resolution studies, i.e. whole tissue Northern blots, in situ hybridization studies indicate widespread expression patterns of EDG-1 and EDG-5 (25, 26). EDG-6, however, shows a hemopoietic-restricted expression pattern (21). Various cell lines in tissue culture express one or more of the EDG receptors. These data raise the question of the role of multiple receptors for SPP.

Several cell systems were tested for endogenous expression of the SPP receptor transcripts. All cell lines tested, namely, HEK293, Jurkat, HEL, HepG2, vascular smooth muscle cells, (fibroblasts and endothelial cells) expressed one or more of EDG-1, -3, or 5. Although some cell lines expressed low levels, for example Jurkat and HepG2 cells, prolonged exposure of Northern blots yielded detectable signals. Also SPP-induced rapid intracellular calcium response has been shown in Chinese hamster ovary-K1 cells, mouse NIH 3T3 cells, monkey COS-1 cells, human bladder carcinoma J82 cells, and rat C6 glioma cells, among others. Indeed, most cell lines tested exhibited biological responses to SPP such as cell rounding, ERK-2 activation, proliferation and inhibition of adenylate cyclase (13, 24, 27).

Thus, establishment of a truly negative heterologous expression system is an important step to molecularly characterize each SPP receptor isotype. In this report, we characterize the SPP receptors in a Xenopus oocyte functional signaling assay by switching the intracellular signaling pathways of the SPP receptors with chimeric Go proteins. Furthermore, we examine the signaling and pharmacological properties of EDG-1, -3, and -5 GPCRs for SPP.

EXPERIMENTAL PROCEDURES

Fatty acid-free bovine serum albumin (fBSA), collagenase type IA, O-phosphorylethanolamine, phosphorylcholine chloride were purchased from Sigma. Sphingosine, dimethylsphingosine, SPP, sphingosylphosphorylcholine (SPC), sphingomyelin, N-acetylsphingosine (C2), N-hexanoylsphingosine (C6), N-palmitoylsphingosine (C16), N-oc-tanoylsphingosine 1-phosphate (C8-P), suramin sodium were purchased from Biomol Research Laboratories Inc. (Plymouth Meeting, PA). Sphingolipids were added to cells as a complex with 0.4% fBSA in OR2 buffer (5 mM Hepes, 1 mM Na2HPO4, 82.5 mM NaCl, 2.5 mM KCl, 0.5 mM CaCl2, pH 7.4). Cap analogue and T7 polymerase are from New England Biolabs (Beverly, MA).

The cDNA encoding the different receptors were cloned by polymerase chain reaction was performed with a PfuTurbo polymerase mix (10×) on human heart cDNA for EDG-3 and on rat heart cDNA for EDG-5. Primers used were 5’-ACTCGAGGCACACTGCCCTTCCGCGGTG-3’ (sense) and 5’-CTTGTAGCAGGTGGATCGAAGATTCC-3’ (antisense) for the human EDG-3 receptor, 5’-AAACTCAGGGCGGTGTGACTGAGTAC-3’ (sense) and 5’-ATCAGTACGTGCAAGACCTGAGATCCAGG-3’ (antisense) for the rat EDG-5 receptor, respectively. EDG-3 and EDG-5 polymerase chain reaction products were subcloned in frame with a N-terminal Flag peptide (DYK-
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RESULTS

We searched for a system that lacks any endogenous response to SPP. As previously reported (30), defolliculated oocytes injected with the photoprotein aequorin constitute a highly sensitive assay for the G_{qi}-linked receptors, inducing rises in intracellular calcium via the second messenger inositol 1,4,5-triphosphate. We confirmed that oocytes contain an endogenous G_{qi}-linked LPA receptor, which induced strong intracellular calcium rises when stimulated with nanomolar concentrations of LPA (32). In contrast, even micromolar concentrations of SPP were inactive suggesting that SPP receptors are not expressed in oocytes or, if present, are unable to couple to the G_{qi} pathway (data not shown). To address these issues, we used the G-protein chimeras G_{aq} and G_{ap}, which couple G_{i} and G_{q}-protein-coupled receptors to the G_{qi}/phospholipase-Cβ pathway, respectively (29). When oocytes were microinjected on the same day with 20 nl of ccRNA sample at 1 nM dihydrosphingosine, 10 μM sphingosine-1-phosphate, 10 μM dimethylsphingosine, 10 μM C2-eradamer, 10 μM C16-eradamer, 10 μM C24-eradamer, 1 μM ceramide-8 1-phosphate, 10 μM sphingomyelin, 1 mM phosphorylcholine, and 1 μM O-phosphorylcholine, or 1 mg/ml suramin, and light emission was integrated for 120 s. Results are mean ± S.E. derived from at least seven oocytes for each agonist concentration.

TABLE I

| Compound         | EDG-1 + G_{qi} | EDG-3   | EDG-5 + G_{aq} |
|------------------|----------------|---------|----------------|
| Sphingosine, 10 μM | <5%            | <5%     | <5%            |
| Dimethylsphingosine, 10 μM | <5%            | <5%     | <5%            |
| Ceramide 2, 10 μM | <5%            | <5%     | <5%            |
| Ceramide 16, 10 μM | <5%            | <5%     | <5%            |
| Ceramide-8 1-phosphate, 500 nM | <5%            | <5%     | <5%            |
| Sphinganine, 10 μM | <5%            | <5%     | <5%            |
| Dihydrospingosine, 10 μM | <5%            | <5%     | <5%            |
| Sphingomyelin, 10 μM | <5%            | ND*     | ND*            |
| Phosphocholine, 1 mM | ND            | <5%     | <5%            |
| Phosphoethanolamine, 1 mM | <5%            | <5%     | <5%            |
| Suramin, 1 mg/ml (700 μM) | <5%            | <5%     | <5%            |
| Sphingosine 1-phosphate | 100% ± 20.85  | 100% ± 12.98 | 100% ± 14.55 |

* ND, not determined.
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Acquorin-injected oocytes expressing EDG-1 + Gαqi (A), EDG-3 (B), or EDG-5 + Gαqi (C) were incubated with test compounds, namely, 10 μM sphingosine (SGO), 10 μM dimethylsphingosine (DMS), 10 μM dihydrosphingosine (DHIS), 10 μM C2-ceramide (C2), 10 μM C6-ceramide (C6), 10 μM C16-ceramide (C16), 1 μM C8-1-phosphate (C8-P), 10 μM sphingomyelin (SM), 1 mM phosphorylcholine (ChoP), 1 mM o-phosphoethanolamine (PEA), or 1 mg/ml suramin (Sura) for 120 s then challenged with 5 nM SPP. Light emission was quantitated as described. Results are mean ± S.E. derived from at least five oocytes.

croinjected with only chimeric Gαqi and Gαpq ccRNAs, allowed to express these proteins, and subsequently stimulated with nanomolar-micromolar SPP, calcium rises were not induced. These data suggest that oocytes do not express any detectable calcium-coupled SPP receptors linked to the Gαqi, Gαpq, or Gαqs pathway.

Next, injection of human EDG-1, human EDG-3, and rat EDG-5 ccRNA, followed by stimulation with SPP was performed. As shown in Fig. 1A, strong intracellular calcium increases were induced upon expression of the EDG-3 receptor and stimulation of the cells with 50 nM SPP. Although weaker, nanomolar concentrations of SPP also induced significant calcium increases in EDG-5-expressing oocytes. In contrast, EDG-1 expression did not yield any SPP-induced calcium responses. To detect the functionality of the human EDG-1 expressed in oocytes, we co-expressed the receptor with the chimeric Gαqi protein or Gαpq and stimulated the cells with SPP. As shown in Fig. 1B, Gαqi but not Gαpq nor Gαpq allowed EDG-1 to couple efficiently to the Gαqi-pathway. As a control for the functional Gαqi chimera, stimulation by 1 μM isoproterenol of oocytes co-expressing the β2-adrenergic receptor and the Gαqi chimera protein induced calcium mobilization (Fig. 1B). Similarly, to determine whether the microinjected Gαqs was overexpressed and indeed functional, we co-expressed EDG-1, -3, and -5, respectively, with the mouse Gαqs. As illustrated in Fig. 1C, potentiation of SPP-induced calcium rise was observed only for the EDG-3 and -5 receptors. Further, immunoblot analysis (Fig. 1C, inset) shows significant expression of the transfected mammalian Gαqs in oocytes. Co-injection of Gαqs ccRNA (but not Gαpq) also greatly potentiated the EDG-5 response to SPP (Fig. 1D). However, EDG-5, in contrast to EDG-1, can couple to the Xenopus Gαq-like protein, albeit less efficiently than EDG-3. Likewise, co-expression of Gαqs with the EDG-3 receptor potentiated the calcium response induced by SPP (data not shown). Thus, acquorin-loaded oocytes expressing EDG-3 alone or EDG-1 and EDG-5 with the Gαqs protein, constitute a sensitive assay to investigate the specific stimulation of SPP (or any agonists) on these receptors.

Dose-response analysis of SPP stimulation of the three receptors was conducted next. As shown in Fig. 2A, SPP stimulated calcium responses with an EC50 of 2.7, 5, and 7.1 nM for EDG-1, -3, and -5 receptors, respectively. The SPP response was also saturable, indicative of a receptor-dependent response. Related sphingolipids as well as the degradation products of SPP catabolism were also tested for potential agonistic effects. As summarized in Table I, sphingosine, dimethylsphingosine, C2-ceramide, C16-ceramide, C8-1-phosphate, sphinganine, dihydrosphingosine, sphingomyelin, phosphocholine, and phosphoethanolamine as well as the poly cyclic compound suramin did not stimulate any of the EDG-1, EDG-3, or EDG-5 receptors. In contrast, sphingosylphosphorylcholine, a related bioactive sphingolipid, stimulated all three receptors.

Next, we tested whether related compounds acted as antagonists for SPP action. Oocytes expressing respective receptors (with chimeric Gαqi for EDG-1 and EDG-5) were pretreated
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with the test compounds for 150 s, followed by stimulation with 5 nM SPP, and calcium responses were quantitated. As shown in Fig. 3, none of the sphingolipids or phosphoethanolamine, the breakdown product of SPP, antagonized any of the EDG-1, -3, or -5 receptors. Suramin, an anionic polycyclic compound that blocks many receptor/ligand interactions including the bioactive lipids such as LPA and SPP, was also tested. As shown in Fig. 3, suramin antagonized SPP activation of EDG-3 receptor but not EDG-1 or EDG-5. Dose response analysis of suramin action is shown in Fig. 4A. Selective antagonism of SPP action on the EDG-3 receptor was observed with an IC$_{50}$ of ~22 µM (0.03 mg/ml). The action of suramin seems directed at receptor/ligand interaction rather than the receptor/G-protein interaction because (i) EDG-5 was not antagonized whether G$_{	ext{q}0}$ was co-injected or not, and (ii) the effect of suramin on oocytes co-expressing G$_{	ext{q}0}$ and EDG-3 are identical to those expressing EDG-3 alone (data not shown). Dose-response analysis of SPP in suramin-treated oocytes shows that it antagonizes EDG-3 with a competitive isotherm (Fig. 4B). These data indicate that suramin acts as a subtype-specific functional antagonist of EDG-3.

**DISCUSSION**

SPP is well accepted as a broad-spectrum bioactive lipid that induces short term as well as long term effects (1–3). However, its mode of action is controversial with respect to the disparate biological actions it mediates. Recent identification of EDG-1 as a high affinity GPCR for SPP has provided a molecular basis for SPP as an extracellular mediator (10, 14, 15). Because EDG-1 is a prototypical member of a subfamily of GPCRs, several laboratories have addressed whether such receptors are also SPP receptors. Indeed, EDG-3 and -5 have been shown recently to mediate responses to low concentrations of SPP in heterologously expressed systems (19). These observations suggest that EDG-1, -3, and -5 are high affinity SPP receptors. The critical question from these recent studies is why multiple receptors exist for SPP. It could be that these three receptors are unique subtypes of SPP receptors that couple to distinct signaling pathways and thereby regulate specific biological responses. Alternatively, these receptors may couple to similar signaling pathways in a redundant manner. Of course these two possibilities are not mutually exclusive. Pharmacological approaches to the study of SPP receptors have been hampered by difficulties in radioligand binding assays, lack of truly negative cell lines and expression systems and lack of subtype-specific agonists or antagonists. In this study, pharmacological properties and signaling characteristics of EDG-1, -3, and -5 SPP receptors are compared.

Our data indicate that albino Xenopus oocytes represent an extremely useful system to analyze the properties of individual SPP receptors. SPP did not induce calcium responses in un.injected or G$_{	ext{q}0}$, G$_{	ext{m}0}$, or G$_{	ext{m}0}$-expressing Xenopus oocytes. However, expression of SPP receptors, EDG-3 and EDG-5, conferred SPP-responsive calcium increases. It is known that oocytes express a G$_{	ext{q}0}$-like protein, and the downstream signaling pathways can be efficiently activated by heterologous expression of G$_{	ext{q}}$-coupled receptors (30). When stimulated by SPP, EDG-3 induced a robust calcium response in oocytes, suggesting that it is a G$_{	ext{q}}$-coupled SPP receptor. Although the EDG-5 response to SPP was detectable, it was much less than EDG-3, suggesting that it coupled less efficiently to the Xenopus G$_{	ext{q}}$. However co-expression of G$_{	ext{m}0}$ or G$_{	ext{m}0}$ potentiated the coupling of EDG-3 and EDG-5 to intracellular calcium rises, suggesting that EDG-3 and -5 may also couple to G$_{	ext{i}}$. EDG-1, on the other hand, was unable to couple to the G$_{	ext{i}}$ pathway, even when overexpressed with the G$_{	ext{m}0}$ proteins. We have previously shown that EDG-1 is a G$_{	ext{q}}$-coupled receptor (33). Thus, as expected, co-expression of EDG-1 with G$_{	ext{m}0}$ allowed ligand-activated coupling to the G$_{	ext{q}}$ pathway. EDG-1-induced calcium increases in other cell lines may be due to a non-G$_{	ext{q}}$ pathway, for example by the activation of phospholipase-C$_{B2}$ by $\beta$y subunits of the G$_{i}$ proteins (34). Indeed, in Chinese hamster ovary cells, EDG-1 mediated calcium responses were inhibited by pertussis toxin (15). These manipulations allowed the efficient testing of the three SPP receptors in the oocyte system, which has no endogenous responses to SPP.

All three SPP receptors were stimulated by SPP with EC$_{50}$ values in the nanomolar range. None of the related lipids activated these receptors as agonists. We have previously shown that the bioactive lipid LPA is a low affinity agonist for the EDG-1 receptor (35). However, a limitation of this system is that we cannot test the efficacy of LPA to activate these receptors because of the presence of endogenous G$_{i}$-coupled LPA receptors in oocytes (32, 36). Nevertheless, we found that the related bioactive sphingolipid mediator, SPC, is a potent activator of SPP receptors. This bioactive lipid mediator induces a variety of effects including cell proliferation, intracellular calcium increases, and wound healing (37, 38). Recently vascular smooth muscle cell calcium increases and mitogen-activated protein kinase activity were shown to be regulated by SPC (39). Whether some or all of SPC responses occur through EDG-1, -3, and -5 remains to be determined.

Using the oocyte system, we also tested whether related lipids acted as antagonists for the EDG-1, -3, and -5 receptors. We found that none of the structurally related sphingolipids, as well as the SPP breakdown product phosphoethanolamine, antagonized these receptors. The polycyclic anionic compound suramin is known to block many ligand receptor interactions including those of LPA and ATP. Suramin was shown to inhibit Rho-dependent neurite retraction induced by SPP in N1E-115 neuronal cells (40). Suramin is also able to inhibit SPP- or LPA-induced invasion of T-lymphoma cells (23). However, it did not inhibit SPP-induced cell proliferation and stress fiber induction in NIH 3T3 cells (9) or tyrosine phosphorylation of p125$^{FAK}$ (a downstream target of Rho) or DNA synthesis induced by SPP in Swiss 3T3 fibroblasts (9). This last observation was compared with the strong inhibition by suramin of LPA-induced tyrosine phosphorylation of p125$^{FAK}$ and DNA synthesis, leading the conclusion of the intracellular signaling action of SPP (9). However, our data indicate that the effect of suramin is receptor subtype specific. Suramin did not block the SPP-induced calcium mobilization by the EDG-1 and the EDG-5 receptors but inhibited the EDG-3 receptor with an IC$_{50}$ of ~22 µM. The differential effect of suramin may be related to heterogeneity of the structure of the receptors, particularly those residues involved in ligand activation and antagonist binding. Further mutagenesis studies should address this issue.

In conclusion, our data show that EDG-1, -3, and -5 receptors are (i) activated by extremely low concentrations of SPP; (ii) activated by SPC; and (iii) capable of coupling the G$_{	ext{m}0}$ protein to the phospholipase-Cl$\beta$ pathway. Further, only EDG-3 and EDG-5 receptors are able to couple to the G$_{i}$ signaling pathway. In addition, the EDG-3 subtype is selectively inhibited by suramin as a functional competitive antagonist. These observations support the notion that distinct SPP receptors are involved in the regulation of specific biological processes by coupling to discrete signaling pathways.

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