EDG1 Is a Functional Sphingosine-1-phosphate Receptor That Is Linked via a $G_{i/o}$ to Multiple Signaling Pathways, Including Phospholipase C Activation, Ca$^{2+}$ Mobilization, Ras-Mitogen-activated Protein Kinase Activation, and Adenylate Cyclase Inhibition*

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In Chinese hamster ovary (CHO) cells transiently transfected with an expression vector for EDG1, but not an empty vector, sphingosine-1-phosphate (SP) at a concentration as low as 10$^{-7}$ M caused an increase in the intracellular free Ca$^{2+}$ concentration ([Ca$^{2+}$]i) as a result of mobilization of Ca$^{2+}$ from both intracellular and extracellular pools. In a CHO clone stably expressing EDG1 receptor (CHO-EDG1 cells), SP induced increases in the production of inositol phosphates and the [Ca$^{2+}$]i and inhibited forskolin-induced increase in the cellular cAMP content, all in a manner sensitive to pertussis toxin. SP also activated mitogen-activated protein kinase in CHO-EDG1 cells in pertussis toxin-sensitive and Ras-dependent manners. To evaluate the spectrum of agonists for EDG1, we used human erythroleukemia (HEL) cells, which at naive state do not respond to SP or structurally related lipids with an increase in the [Ca$^{2+}$]i. In HEL cells stably expressing EDG1 receptor (HEL-EDG1 cells), SP dose-dependently increased the [Ca$^{2+}$]i with half-maximal and maximal concentration values of 10$^{-9}$ and 3 × 10$^{-7}$ M, respectively; sphingosylphosphorylcholine at exclusively high concentrations, but not sphingosine at all, also increased the [Ca$^{2+}$]i. HEL-EDG1 cells bound $^{32}$P-labeled SP, which was displaced dose dependently by unlabeled SP. These results indicate that EDG1, a member of the EDG family G protein-coupled receptors, is a specific, high-affinity SP receptor.

Recent studies (1–3) provide increasing evidence of roles for lysosphingolipids as mediators to elicit a variety of physiological and pathophysiological responses. Thus, the lysosphingolipids SP$^1$ and SPC have been shown to evoke diverse cellular responses in various cell types, including mitogenesis (1, 2), inhibition of migration (4, 5), cell shape change (6), and microfilament reorganization (6, 7). Stimulation of cells with the lysosphingolipids triggers the activation of multiple intracellular signaling molecules, including phospholipase C (2, 5, 8, 9), phospholipase D (8), PKC (10), MAPK (5, 11), and K$^+$ channel (muscarinic K$^+$ current) (12). Many of the lysosphingolipid-induced responses are demonstrated to be inhibited by PTX pretreatment (5, 8–13). In addition, either an increase or a decrease in cellular cAMP content in response to SP has been reported, depending on cell types used (5, 13). These observations suggest the existence of multiple G protein-coupled cell surface receptors for SP and SPC.

Recently, the orphan G protein-coupled receptor EDG2 was identified as a functional receptor for LPA (14). Moreover, EDG4 was very recently identified to be the second LPA receptor (15). EDG2 and EDG4 are members of the EDG family of receptors comprising EDG1 (16), EDG3 (17), and AGR16 (18)/H218 (19), which have 36–58% homology in amino acid sequences with each other. SP is related in its structure to LPA, and in some cell types, LPA and SP have been suggested to share a cell surface receptor (20, 21). These observations prompted us to examine the possibility that members of the EDG family receptors could function as a receptor for the lysosphingolipids. Many of cell lines usually used for expression of exogenous genes, including COS, NIH3T3 and HEK293 cells, respond to SP (13), which hampered expression cloning of SP receptor gene and functional analysis of cloned SP receptor gene. In the present study, by using carefully selected mammalian cell expression systems, we found that EDG1 is a functional receptor with a high specificity and affinity for SP. We demonstrate that EDG1 is coupled via a $G_{i/o}$ protein to multiple effector pathways, including phospholipase C, adenylate cyclase, and Ras/MAPK.

MATERIALS AND METHODS

Cells—CHO-K1(CHO) and HEL cells, obtained from RIKEN Cell Bank and the Japanese Cancer Research Resources Bank (Tokyo, Japan), respectively, were grown in Ham’s F-12 (CHO) and RPMI (HEL) media supplemented with 10% fetal calf serum (Equitech-Bio, Ingram, TX), 100 units/ml penicillin, and 100 μg/ml streptomycin (Wako Pure Chemicals, Osaka, Japan). Before each experiment, cells were switched to the respective medium supplemented with 1% fetal calf serum.

Cells were transfected with constructs for human EDG1 cDNA using the calcium phosphate DNA precipitation method.Transiently transfected CHO cells were used for all experiments. Stable CHO-EDG1 cell lines were generated by transfection of CHO-K1 cells and G418 selection (Bioxytech). Western blot analysis confirmed the expression of the EDG1 receptor in the stable cell lines as described (20).

Chemicals, Osaka, Japan). Before each experiment, cells were switched to the respective medium supplemented with 1% fetal calf serum.
Measurements of \([\text{Ca}^{2+}]\)i, Inositol Phosphate Production, and cAMP Content—The \([\text{Ca}^{2+}]\)i was measured as described previously (22) in fura-2-loaded, trypsinized cells with a CAF-110 spectrofluorimeter (Japan Spectroscopy, Inc., Tokyo, Japan) with excitation at 340 and 380 nm and emission at 500 nm. For measurements of production of inositol phosphates, cells were prelabeled with 4 \(\mu\text{Ci/ml}\) myo-[2-3H]inositol in Ham’s F-12 medium supplemented with 1% fetal calf serum for 24 h, and stimulated with SP in the presence of 10 \(\mu\text{M}\) LiCl. The fraction containing total inositol phosphates was separated as described in detail elsewhere (22), and the radioactivity was quantitated by scintillation counting. For measurements of cAMP, cells were first incubated with 0.2 \(\mu\text{M}\) forskolin for 5 min and then stimulated with SP for a further 5 min. The reaction was terminated by adding HCl (a final concentration of 0.1 \(\mu\text{M}\)), and the amount of cAMP in the acid extracts was measured by radioimmunoassay using a Yamasa (Choshi, Japan) cAMP assay kit (22).

Plasmids, Transfection, and MAPK Assay—Rat full-length EDG1 cDNA was cloned by hybridization screening of a Agt10 cDNA library made from rat lung (18, 22) by using as a probe a 365-bp (589–954) human EDG1 cDNA fragment, which was obtained by PCR amplification of a human umbilical vein endothelial cell cDNA library. The 1.7-kbp StuI-BglII fragment of EDG1 cDNA was adaptor ligated into mammalian expression vector pCAGGS (kindly donated by Dr. J. Miyazaki, Osaka University, Osaka, Japan) (23) at the BstXI site downstream of the CAG promoter. Transfections were carried out by using LipofectAMINE (Life Technologies, Inc.) as described (24). Stable transfecants were selected with G418 as described (22). An expression vector for EDG1 with a C-terminal HA tag was created by a PCR-based method (25) using the sense primer 5’-AGTTGGCGCTATGGTGTC-
CHO-EDG1 cells prelabeled with myo-$\alpha$ EDG1 (CHO-EDG cells), both in a PTX-sensitive manner. With fura-2, and stimulated with 10$^{-6}$ M SP for 60 min in the presence of 10 mM LiCl. b, The CHO-EDG1 cells were pretreated with 100 ng/ml PTX or not pretreated for 24 h, loaded with fura-2, and stimulated with 10$^{-7}$ M SP or 3 × 10$^{-6}$ M ATP. The data are expressed as means ± S.E. of three determinations.

Fig. 3. SP induces production of inositol phosphates (a) and an increase in the [Ca$^{2+}$], in CHO cells stably expressing EDG1 (CHO-EDG cells), both in a PTX-sensitive manner. a, The CHO-EDG1 cells prelabeled with myo-$\alpha$-H]inositol were pretreated with 100 ng/ml PTX or not pretreated for 24 h and stimulated with 10$^{-6}$ M SP for 60 min in the presence of 10 mM LiCl. b, The CHO-EDG1 cells were pretreated with 100 ng/ml PTX or not pretreated for 24 h, loaded with fura-2, and stimulated with 10$^{-7}$ M SP or 3 × 10$^{-6}$ M ATP. The data are expressed as means ± S.E. of three determinations.

CTCCACACAGATCCA-3' and the antisense primer 5'-TTAAGCGTATCTGGAACATCGTATGGGTAAGAAGAATTGACGTTTCCAG-AAGACATATAA-3'. Expression of HA-tagged EDG1 protein was examined by Western blot analysis using monoclonal anti-HA antibody (Boehringer Mannheim) (24, 26). For measurements of MAPK activity in transiently transfected cells, CHO cells in 35-mm dishes were co-transfected with pME18S-Myc-ERK (24) and either pME18S-Asn17Ras (24, 26) (Asn17-AH-Ras cDNA was obtained from Dr. G. Cooper, Harvard Medical School, Boston, MA) or an empty vector pME18S (22, 24, 26). Myc-tagged MAPK was immunoprecipitated by using a mouse monoclonal anti-Myc epitope antibody (clone 9E10). MAPK activity associated with the immune complex was assayed in vitro using myelin basic protein (Sigma) as substrate, as described (24). The band shift of endogenous p42ERK2 in CHO cells stably expressing EDG1 was detected by Western blot analysis of total cell lysate with a mouse monoclonal anti-ERK antibody (clone 03–6600; Zymed Laboratories Inc.). Bovine G$\alpha_q$ cDNA (27) was obtained from Dr. H. Bourne (University of California, San Francisco, CA) and ligated into the mammalian expression vector pME18S.

$^{[32P]}$SP Binding—Cells on a 12-well plate were washed with a binding medium consisting of RPMI 1640 medium containing 10 mM Hepes (pH 7.4) and 0.1% fatty acid-free bovine serum albumin (Sigma) and incubated in the same medium containing freshly prepared 5 × 10$^{-15}$ M $^{[32P]}$SP (88,000 cpm/pmol) in the presence or absence of varied concentrations of unlabeled SP or 10$^{-6}$ M related lipids for 30 min at 25 °C. $^{[32P]}$SP was prepared as described (7, 21). Time course studies revealed that $^{[32P]}$SP binding to cells became saturated by 30 min. After washing cells three times with ice-cold binding medium, cell-bound radioactivity was counted. Specific binding was determined by subtracting nonspecific binding in the presence of 3 × 10$^{-6}$ M SP from each binding value.

Northern Blot Analysis—Isolation of total RNA and enrichment of poly(A)$^+$ RNA were performed as described previously (18, 22). RNA was separated by 1.0% agarose-formaldehyde gel electrophoresis, transferred onto a nylon membrane (GeneScreen, DuPont NEN), and hybridized with cDNA probes labeled with $[^3P]$dCTP (DuPont NEN) by the random priming method (22). The cDNA probes used were as follows: 0.95-kbp EcoRV-Smal fragment of rat EDG1 coding the entire transmembrane regions, 365-bp fragment of human EDG1 coding the entire transmembrane and its entire C-terminal region.

Materials—SP, SPC, and ceramide (C8)-1-phosphate were obtained from Biomol (Plymouth Meeting, PA). Sphingosine, lysophosphatidylserine (purified from bovine brain), lysophosphatidylcholine (C18), lysophosphatidylethanolamine (C18), ceramide (C2, C6, and C18), PA
(diC18), LPA (C18), and glucosylsphingosine were purchased from Sigma. LysoPI (purified from bovine liver) was purchased from Avanti (Birmingham, AL). Fura-2/AM solution was purchased from Dojin (Kumamoto, Japan). U73122 was purchased from Research Biochemicals International (Natik, MA). Rabbit polyclonal anti-Ga1 antibody (C10) and rabbit polyclonal anti-Gaq/11 antibody (C19) were purchased from Santa Cruz Biochemicals (Santa Cruz, CA). SP was dissolved in dimethylsulfoxide at 2 \times 10^{-3} M, aliquoted, and stored at -20 °C. Other lipids were dissolved in methanol. Final solvent concentrations did not exceed 0.1%.

RESULTS AND DISCUSSION

We first used CHO cells for transient transfection experiments, because we found that CHO cells had an undetectable level of endogenous EDG1 mRNA (Fig. 1). In CHO cells transiently transfected with an expression vector for HA-tagged EDG1, SP at 10^{-10} M caused a biphasic, sustained increase in the [Ca^{2+}]i with the peak [Ca^{2+}]i increment of 110 \pm 9 nM (mean \pm S.E.; n = 3), as evaluated as an averaged [Ca^{2+}]i increase in a cell population (Fig. 2a). In the absence of extra-
cellular Ca^{2+} (0 mM Ca^{2+} plus 0.1 mM EGTA), SP still elicited a transient increase in the [Ca^{2+}]i, although the amplitude of the peak [Ca^{2+}]i increase was diminished compared with that in the presence of extracellular Ca^{2+}, and the sustained plateau phase of the [Ca^{2+}]i increase was abolished. By contrast, in CHO cells transfected with an empty vector, SP at this concentration did not induce a significant increase in the [Ca^{2+}]i. EDG1 receptor protein in EDG1-transfected CHO cells was detected by Western blot analysis using anti-HA antibody (Fig. 2b).

Because many Ca^{2+}-mobilizing receptor agonists are known to activate MAPK/ERK, we examined whether SP activated MAPK in EDG1-transfected CHO cells. We co-transfected CHO

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**Fig. 5.** A phospholipase C inhibitor inhibits SP-induced inositol phosphate production (a) and [Ca^{2+}]i increase (b). a, the CHO-EDG1 cells prelabeled with myo-[2-3H]inositol were pretreated with 10^{-5} M phospholipase C inhibitor U73122 or left untreated for 5 min and then stimulated with 10^{-6} M SP for 60 min in the presence of 10 mM LiCl. b, the CHO-EDG1 cells were loaded with fura-2, pretreated with U73122, or left untreated, and then stimulated with 10^{-6} M SP. The data are expressed as means \pm S.E. of three determinations.

**Fig. 6.** SP activates MAPK in manners sensitive to PTX and genistein and dependent on Ras. a, CHO-EDG1 cells were pretreated with either PTX (100 ng/ml for 24 h), genistein (100 \mu M for 60 min), phorbol-12,13-dibutyrate (PDBu, 1 \mu M for 24 h) or GF109203X (3 \mu M for 10 min) and then stimulated with 10^{-6} M SP for 5 min. Cell lysate was separated on 10% PAGE, followed by Western blotting using anti-MAPK (ERK2) antibody. b, CHO-EDG1 cells were transiently transfected with Myc-tagged MAPK expression vector and either an Asn17-H-Ras expression vector or an empty vector and stimulated with 10^{-6} M SP for 5 min. Representative autoradiograms of phosphorylated myelin basic protein (MBP) and anti-Myc epitope (Myc-MAPK) immunoblots are shown on the top of the bar graph as described for Fig. 1. Note that the expression levels of Myc-tagged MAPK were similar between the two transfection groups. The data are expressed as means \pm S.E. of three determinations.
cells with an expression vector for Myc-tagged MAPK and either the EDG1 expression vector or the empty vector. We then stimulated these cells with SP (10^{-8} M), immunoprecipitated Myc-tagged MAPK using anti-Myc epitope antibody, and measured the in vitro MAPK activity using myelin basic protein as substrate. SP induced a 25-fold increase in the MAPK activity in cells transfected with the EDG1 expression vector, compared with only a 2-fold increase in cells transfected with the empty vector (Fig. 2c). The two transfection groups showed equal expression levels of Myc-tagged MAPK, as evaluated by anti-Myc epitope immunoblot analysis (Fig. 2c).

We established a CHO clone stably expressing EDG1 receptor (CHO-EDG1) for further analysis. In CHO-EDG1 cells, but not in parental CHO cells, SP (10^{-6} M) stimulated inositol phosphate production 2-fold. Pretreatment of CHO-EDG1 cells with PTX (100 ng/ml for 24 h) inhibited SP-induced inositol phosphate production by 80% (Fig. 3a). PTX pretreatment also strongly inhibited SP (10^{-7} M)-induced [Ca^{2+}], increase in CHO-EDG1 cells (446 ± 20 versus 42 ± 3 nM in control and PTX-pretreated cells, respectively) (Fig. 3b). In contrast, PTX pretreatment did not inhibit ATP-induced, P2 receptor-mediated [Ca^{2+}], increase (456 ± 7 versus 414 ± 8 nM in control and PTX-pretreated cells, respectively). Similarly, in human erythroleukemia HEL cells stably expressing EDG1 (see below), SP-induced [Ca^{2+}], increase was PTX-sensitive. As shown in Fig. 4a, both CHO cells and HEL cells express G_{oq11} as well as G_{qq}, indicating that dominant coupling of EDG1 via G_{q} to phospholipase C and Ca^{2+} mobilization is not attributable to a low-level expression of G_{oq11}. To test a role for G_{q} subunits of G_{q} protein in SP-induced Ca^{2+} mobilization, we examined the effect of the overexpression of G_{q}, a G_{q} scavenger (27), on SP-induced Ca^{2+} mobilization. The expression of G_{q} substantially (75%) reduced SP-induced [Ca^{2+}], increase compared with the vector control (Fig. 4b). In contrast, ATP-induced [Ca^{2+}], increase, which was PTX insensitive, was little inhibited by the expression of G_{q}. These results are consistent with the notion that G_{q} subunits mediate SP-induced increase in the [Ca^{2+}],. We also examined the relation between phospholipase C activation and Ca^{2+} mobilization in SP-stimulated CHO-EDG1 cells. Pretreatment of CHO-EDG1 cells with U73122 (10 µM), a phospholipase C inhibitor, nearly totally suppressed SP-induced inositol phosphate production (Fig. 5a). U73122 also inhibited SP-induced [Ca^{2+}], increase (Fig. 5b), suggesting that SP mobilizes Ca^{2+} from intracellular pools through the action of inositol-1,4,5-triphosphate.

In CHO-EDG1 cells, SP (10^{-8} M) activated endogenous MAPK, as evaluated by detection of the band shift of p42 ERK2 (Fig. 6a). Pretreatment of the cells with PTX totally abrogated SP-induced MAPK activation (Fig. 6a). The addition of genistein, a tyrosine kinase inhibitor, or the expression of a dominant negative form of Ras (Asn^{17}-H-Ras) (24, 26) also strongly inhibited SP-induced MAPK activation by 60 and 83%, respectively (Fig. 6, a and b). In contrast, SP-induced MAPK activation was for the most part resistant to down-regulation of PKC by prolonged pretreatment of cells with phorbol-12,13-dibutyrate (1 µM for 24 h) (Fig. 6a). The same procedure abrogated MAPK activation in response to acute stimulation with phorbol-12,13-dibutyrate, implying that down-regulation-sensitive isoforms of PKC were effectively depleted (data not shown). The specific PKC inhibitor GF109203X (3 µM) failed to inhibit SP-induced MAPK activation. GF109203X completely inhibited PDBu-induced MAPK activation (Fig. 6a).

Because SP-induced [Ca^{2+}], increase and MAPK activation was sensitive to PTX, we examined whether SP decreased the cellular cAMP content via a PTX-sensitive G protein. As shown in Table I, SP reduced the forskolin-stimulated increase in the cAMP content by 46% in CHO-EDG1 cells. Pretreatment of cells with PTX (100 ng/ml for 24 h) totally abolished this effect of SP. SP was without effect on the cAMP content in parental CHO cells.

In parental CHO cells, SP at higher concentrations (≥10^{-8} M) slightly increased the [Ca^{2+}], probably because of a low level of the expression of endogenous SP receptor(s). Northern analysis of CHO mRNA (Fig. 1) reveals that CHO cells express a low level of mRNA of the putative SP receptor AGR16 (29), but not of EDG1, or EDG3, another putative SP receptor (29). In addition, CHO cells show a marked [Ca^{2+}], increase response to a structurally related lipid LPA. To determine agonist specificity of EDG1, it was necessary to use a cell line that does not respond to either SP or related lipids. We found that HEL cells satisfied these criteria (Fig. 7a), whereas other cell lines commonly used for transfection, including COS7 and HEK293, showed considerable background signals in response to SP, as reported by others (13). Consistent with this, HEL cells detectably expressed none of transcripts of EDG1, EDG3, or AGR16 (Fig. 1). HEL cells also did not detectably express mRNA of the LPA receptors EDG2 or EDG4. We established a HEL cell clone stably expressing EDG1 (HEL-EDG1). In HEL-EDG1 cells, SP increased the [Ca^{2+}], dose dependently, with EC_{50} and maximal concentration values of 10^{-9} to 3 × 10^{-7} M, respectively (Fig. 7b). SPC was a much less potent agonist, with EC_{50} value of 10^{-7} M, although the maximal response obtained with SPC at 10^{-6} M was comparable to that induced by SP. LPA, PA, and LysoPI induced small increases in the [Ca^{2+}], (15–20% of the maximal SP-induced response). However, LPA-induced [Ca^{2+}], increase was not abolished by the previous addition of SP (10^{-8} M), unlike the SPC-induced [Ca^{2+}], response, which was inhibited by the previous addition of SP but not of prostaglandin E2 (Fig. 7c). Similarly, PA- and LysoPI-induced [Ca^{2+}], responses were not inhibited by SP. These results may suggest that the [Ca^{2+}], increase induced by LPA and presumably PA and LysoPI were mediated by endogenous LPA receptors but not by EDG1. Consistent with this, HEL-EDG1 cells express a slight level of EDG4 transcript (Fig. 1). However, we cannot conclusively rule out the possibility that LPA, PA, and LysoPI act as very weak agonists for EDG1. Other lipids examined, which include sphingosine, sphingomyelin, ceramide (C2, C8, and C18), ceramide-1-phosphate, glucosylsphingosine, lysophosphatidylcholine, lysophosphatidylethanolamine, and lysophosphatidylserine, up to 10^{-4} M did

### Table I

| Cells          | Pretreatment | Stimulation | cAMP content (pmol/cell) |
|---------------|--------------|-------------|-------------------------|
| CHO           | None         | None        | 0.62 ± 0.05 NS          |
|               | Forskolin    | None        | 9.65 ± 0.37 NS          |
|               | Forskolin + SP| None        | 6.31 ± 0.43 NS          |
|               |               | FTX         | 6.31 ± 0.07 NS          |
| CHO-EDG1      | None         | None        | 0.67 ± 0.01 p < 0.05a  |
|               | Forskolin    | None        | 6.40 ± 0.12 p < 0.05a  |
|               | FTX          | None        | 0.68 ± 0.01 NS          |
|               | Forskolin + SP| None        | 8.15 ± 0.15 NS          |
|               | FTX          | FTX         | 8.00 ± 0.07 NS          |

*Statistically significant by Student’s t test.*
not increase the $[\text{Ca}^{2+}]_i$ in HEL-EDG1 cells.

We detected a specific binding of $[^{32}\text{P}]\text{SP}$ to HEL-EDG1 cells, which was inhibited dose dependently by the addition of unlabeled SP with an IC$_{50}$ value of $5 	imes 10^{-8} \text{M}$ (Fig. 8a). In contrast, we did not detect a significant, specific binding of $[^{32}\text{P}]\text{SP}$ to parental HEL cells. We examined competition of $[^{32}\text{P}]\text{SP}$ binding to HEL-EDG1 cells by related lipids. SPC at $10^{-6} \text{M}$ reduced $[^{32}\text{P}]\text{SP}$ binding (Fig. 8b). Other related lipids examined did not compete with SP.

In the present study, we characterized SP-induced signaling pathways in CHO and HEL cells that had been transiently or stably transfected with the EDG1 expression vector. The transient expression of EDG1 in CHO cells conferred responsiveness to SP in terms of $\text{Ca}^{2+}$ mobilization (Fig. 2a) and MAPK activation (Fig. 2b). In contrast, in vector-transfected controls SP at the dose examined did not elicit a response. Further examinations in CHO cells stably expressing EDG1 (CHO-EDG1) revealed that SP stimulation induced the production of inositol phosphates (Fig. 3a) and inhibition of cAMP accumu-
mediate responses to SP in Jurkat T cells or EDG1. The EC_{50} value (10^{-9} M) for the SP-induced [Ca^{2+}]_{i} response in HEL-EDG1 cells is very close to those reported for SP-induced [Ca^{2+}]_{i} increase in HEK293 cells (13) and for SP-induced activation of muscarinic K^{+} current in atrial myocytes (12) but is much lower than those reported in Swiss 3T3 cells for SP-induced mitogenesis, [Ca^{2+}]_{i} increase, and MAPK activation (2, 8, 11). In HEL-EDG1 cells (present study) and HEK293 cells (13), SPC is much less potent than SP in increasing the [Ca^{2+}]_{i}, whereas in atrial myocytes SPC is nearly equipotent to SP (12). Thus, it appears that there exist multiple SP receptor subtypes that are distinct from EDG1 (30). With respect to this, An et al. (29) reported very recently that EDG3 and AGR16 function as SP receptors, as evaluated by the serum response element-driven reporter system and Ca^{2+} flux from Xenopus oocytes. They did not report, however, the sensitivity of these responses to PTX or relative potencies of SP and related lipids as agonists for EDG3 and AGR16.

While this manuscript was in review processes, two papers (31, 32) appeared that reported that EDG1 is an SP receptor. Lee et al. (31) reported that HEK293 cells stably transfected with an EDG1 expression vector showed Rho-dependent cell aggregation in response to SP in a semisolid culture system. They also showed PTX-sensitive MAPK activation on stimulation with SP and high-affinity binding of [32P]SP to HEK293 cells stably expressing EDG1. However, they did not examine the coupling of EDG1 to other effector pathways, i.e., stimulation of the phospholipase C-Ca^{2+} axis, and inhibition of adenylate cyclase. The other paper by Zondag et al. (32) reports that insect Sf9 cells transfected with a baculovirus-encoding EDG1 showed a decrease in the cellular cAMP content in response to SP. Differently from the present study, however, SP failed to increase the [Ca^{2+}]_{i} in Sf9 cells expressing EDG1. They also showed in COS7 cells transiently transfected with an EDG1 expression vector that SP, but not SPC, at the concentration of 10^{-6} M induced activation of MAPK, although they did not measure [Ca^{2+}]_{i} or inositol phosphate production. On the other hand, An et al. (29) reported that EDG1 did not mediate responses to SP in Jurkat T cells or Xenopus oocytes.

In summary, the present study establishes by using mammalian cell expression systems with a low background signal that EDG1 is coupled to concurrent activation of multiple effector pathways via a G_{i/o} protein, including Ras-MAPK, Ca^{2+} mobilization with the activation of phospholipase C, and inhibition of adenylate cyclase (Figs. 2–6). We also determined the relative [Ca^{2+}]-mobilizing potencies of a panel of structurally related compounds in HEL-EDG1 cells (Fig. 7), which reveals that SPC at exclusively higher concentrations can activate EDG1.

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