0026-895X/99/050787-08$3.00/0

MOLECULAR PHARMACOLOGY, **55:**787–794 (1999).

Transduction of Intracellular Calcium Signals through G Protein-Mediated Activation of Phospholipase C by Recombinant Sphingosine 1-Phosphate Receptors

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Received September 23, 1998; accepted February 3, 1999

This paper is available online at http://www.molpharm.org

ABSTRACT

Sphingosine 1-phosphate (S1P) increases intracellular Ca^{2+} concentration in many cell types, but the signaling mechanism remains uncertain. The recent identification of three closely related seven-transmembrane domain receptors for S1P, termed Edg1, H218, and Edg3, support the extracellular ligand role of S1P and allowed examination of Ca^{2+} responses mediated specifically by each receptor subtype. To substantiate each subtype in S1P-induced Ca^{2+} responses and to study the transductional mechanisms, we applied the aequorin luminescence method and the fura-2 fluorescence method in two transfected mammalian cell systems. We showed that H218 and Edg3 were capable of mediating S1P-induced mobilization of intracellular Ca^{2+} when transiently transfected in human TAg-Jurkat T cells. Ca^{2+} responses mediated by Edg1 in TAg-Jurkat cells required coexpression of the G_{qi5} chimeric G protein that links G_i-coupled receptors to G_q. When H218 and Edg3 were stably expressed in rat HTC4 hepatoma cells, S1P induced Ca^{2+} responses with nanomolar EC_{50} values. Edg3, but not H218, elicited a sustained influx of extracellular Ca^{2+}. The coincident formation of inositol phosphates and the complete inhibition of Ca^{2+} responses by the phospholipase C inhibitor U73122 indicated that H218 and Edg3 mobilized Ca^{2+} through activation of phospholipase C. Partial inhibition of Ca^{2+} responses and inositol phosphates formation by pertussis toxin implied that H218 and Edg3 transduce phospholipase C activation and Ca^{2+} responses only partially through G_i proteins. Although these results did not dismiss that S1P may function as an intracellular second messenger in other settings, they definitively proved that S1P can mobilize Ca^{2+} as an extracellular ligand for G protein-coupled receptors.

Several types of lysosphingolipids regulate cell growth, differentiation, and programmed cell death (Spiegel and Merrill, 1996; Meyer zu Heringdorf et al., 1997). Sphingosine 1-phosphate (S1P) is a potent mediator of mitogenesis (Zhang et al., 1991; Goodemote et al., 1995; Berger et al., 1996), cell motility and tumor cell invasiveness (Sadahira et al., 1992; Bornfeldt et al., 1995), platelet activation (Yatomi et al., 1997a), and neurite retraction (Postma et al., 1996). Cell signaling by S1P evokes activation of mitogen-activated protein kinases (Wu et al., 1995), stimulation of $I_{k(Ach)}$ (Bünemann et al., 1996; van Koppen et al., 1996), and prominent increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Zhang et al., 1991; Durieux et al., 1993; Chao et al., 1994; Ghosh et al., 1994; Mattie et al., 1994; Meyer zu Heringdorf et al., 1996; Okajima et al., 1996; van Koppen et al., 1996; Törnquist et al., 1997; Yatomi et al., 1997b). Despite extensive observations in various cell types, the mechanism by which S1P mobilizes intracellular Ca^{2+} is controversial. Some studies suggested that S1P acts as an intracellular second messenger for Ca^{2+} release directly from endoplasmic reticulum (ER) through an inositol trisphosphate (IP_3)-independent mechanism (Ghosh et al., 1994; Mattie et al., 1994). However, the specific molecular target for S1P on ER membranes, presumably a Ca^{2+} channel, has not been identified.

Other studies demonstrated that exogenous S1P increases $[Ca^{2+}]_i$ via cell surface G protein-coupled receptors (GPCRs) that activate phospholipase C (PLC) (Bornfeldt et al., 1995; Okajima et al., 1996, 1997; Im et al., 1997; Noh et al., 1998). In support of an extracellular action of S1P, cDNAs encoding three closely related seven-transmembrane-domain receptors, termed Edg1, H218, and Edg3 (Edg, endothelial differentiation gene), have recently been identified (An et al., 1997; Lee et al., 1998; Zondag et al., 1998). Two of these receptors, H218 and Edg3, mediated S1P-induced serum response element (SRE)-driven transcription in Jurkat cells and trig-

This work was supported in part by a research grant from the American Heart Association (Grant-in-Aid 96007190 to S.A.).

ABBREVIATIONS: S1P, sphingosine 1-phosphate; Edg, endothelial differentiation gene; LPA, lysophosphatidic acid; SP, sphingosine; dHS1P, dihydro-sphingosine 1-phosphate; dMSP, *N,N*-dimethyl-sphingosine; dHSP, dihydro-sphingosine; C6, C6-ceramide; PS, psychosine; G protein, guanine nucleotide-binding protein; GPCR, G protein-coupled receptor; $[Ca^{2+}]_i$, intracellular calcium concentration; PLC, phospholipase C; IP_3, inositol 1,4,5-trisphosphate; PTX, pertussis toxin.

gered Ca^{2+} efflux in *Xenopus* oocytes (An et al., 1997). The other receptor, Edg1, induced cell-cell aggregation through enhanced cadherin expression, inhibited adenylyl cyclase, and activated mitogen-activated protein kinases via G_i protein (Lee et al., 1998; Zondag et al., 1998). However, S1P-induced mobilization of intracellular Ca^{2+} has yet to be attributed to these cloned receptors expressed in mammalian cells and, therefore, the signaling mechanism of S1P-induced Ca^{2+} responses remains uncertain.

In the present study, we expressed these cloned S1P receptors in two types of cultured mammalian cells and measured their abilities to transduce S1P-induced increases in $[Ca^{2+}]_i$ by using two different methods. We demonstrated that H218 and Edg3, when transiently transfected in human TAg-Jurkat T cells or stably transfected in rat HTC4 hepatoma cells, were capable of mobilizing intracellular Ca^{2+}. Furthermore, kinetic characteristics and biochemical signaling properties of H218- and Edg3-mediated Ca^{2+} mobilization were elucidated in the HTC4 stable transfectants.

Materials and Methods

Chemicals. S1P, sphingosine (SP), dihydro-sphingosine 1-phosphate (dHS1P), dihydro-sphingosine (dHSP), *N,N*-dimethyl-sphingosine (dMSP), psychosine (PS), and C6-ceramide (C6) were obtained from Biomol (Plymouth, PA). Sphingosylphosphorylcholine (SPC) was from Matreya, Inc. (Pleasant Gap, PA). 1-Oleoyl-2-hydroxy-*sn*-glycero-3-phosphate (LPA) was from Avanti Polar Lipid (Alabaster, AL). Fatty acid-free human serum albumin was purchased from Sigma (St. Louis, MO). SuperFect transfection reagent for transient transfection was from Qiagen (Hilden, Germany). LipofectAmine, OPTI-MEM medium, and Geneticin (G418 sulfate) were from Gibco-BRL (Gaithersburg, MD). Jurkat leukemic T cells containing the SV40 virus large T antigen (TAg-Jurkat) were obtained from Dr. J. Crabtree (Stanford University). Rat hepatoma HTC4 cells, cell culture media, and fetal bovine serum were from University of California-San Francisco Cell Culture Facilities. An apoaequorin expression construct targeted to cytoplasm (cytAEQ/pcDNA1), coelenterazine *f*, and fura-2/AM were from Molecular Probes (Eugene, OR). A G protein chimera, G_{qi5}, was kindly provided by Dr. B. Conklin (Gladstone Institute, University of California, San Francisco). U73122, U73343, SK&F96365, and pertussis toxin (PTX) were purchased from CalBiochem (La Jolla, CA). *myo*-[^{3}H]Inositol with specific activity 80 Ci/mmol was from Amersham (Arlington Heights, IL). Dowex AG-1X8 resin (200–400 mesh) was obtained from Bio-Rad (Richmond, CA). The enzyme-linked cAMP assay kit was from PerSeptive Biosystems (Framingham, MA).

Transient Transfection of TAg-Jurkat Cells and Measurement of cytoplasmic Ca^{2+} Using Aequorin Luminescence Method. TAg-Jurkat T cells were cotransfected with the apoaequorin expression plasmid (cytAEQ/pcDNA1) and receptor cDNA constructs, at a 1:5 ratio, using SuperFect reagent. The sequences of expression plasmid for the human Edg1, rat H218, and human Edg3 (Edg1/EF3, H218/EF3, and Edg3/EF3, respectively) and the control vector pcDEF3 were previously reported (An et al., 1997). After 10 h of transfection incubation in OPTI-MEM medium, cells were washed once with RPMI 1640 and incubated with 5 μM coelenterazine *f* in RPMI 1640 for 2 h at 37°C. Cells were then washed twice and resuspended in PBS (containing 1 mM $CaCl_2$) at 2×10^6 cells/ml. Light emission resulting from Ca^{2+} binding to the apoaequorin-coelenterazine complex was recorded by luminometry. To obtain kinetic data, aliquots of 2×10^5 cells in 0.1 ml were transferred into a 96-well plate that was placed in an EG&G Berthold microplate luminometer (model LB96V; Berthold, Natick, MA). S1P and other phospholipids dissolved in 0.1 ml of PBS containing 0.1 mg/ml of fatty acid-free human serum albumin were added to the cells through an automated injector. Light emission was recorded every 3 s for 60 s, beginning immediately after the injection. To obtain integrated luminometric data, a Turner Designs 20/20 luminometer (Mountain View, CA) was used to record light emission for 15 s, beginning immediately after injection of S1P.

Stable Transfection of HTC4 Cells and Measurement of $[Ca^{2+}]_i$ Using Fura-2 Fluorescence Method. Rat hepatoma HTC4 cells were transfected with receptor cDNA expression constructs H218/EF3, Edg3/EF3, or control vector pCDEF3 containing the geneticin-resistance gene using LipofectAmine. Selection for stable transfectants was begun 2 days after transfection, using 10% fetal bovine serum-supplemented DME medium containing 500 μg/ml of geneticin. Geneticin-resistant colonies were isolated after an additional 14 days and further cloned through limiting dilution. Levels of specific *h218* or *edg3* mRNA in each HTC4 cell transfectant were assessed by Northern blot analyses. Cell lines with comparable levels of *h218* and *edg3* mRNA were selected for further studies.

Quantification of $[Ca^{2+}]_i$ and $[cAMP]_i$ in HTC4 cell transfectants expressing each subtype of S1P receptor was performed as previously described (An et al., 1994). For $[Ca^{2+}]_i$ assays, HTC4 transfectants were cultured overnight in 100-mm dishes at about 75% confluency. Cells were trypsinized, washed, and loaded with 2.5 μM fura-2/AM in PBS (containing 1 mM $CaCl_2$) for 40 min at 37°C in the dark, washed again, and resuspended in PBS. Cuvettes containing 1×10^6 fura-2-loaded cells in 1.5 ml PBS were positioned in a Perkin Elmer LS 50B fluorometer. Fluorescence was recorded before and after the addition of S1P and other phospholipids dissolved in PBS with 0.1 mg/ml fatty acid-free human serum albumin. The values of $[Ca^{2+}]_i$ (in nanomolar) were calculated from the ratio of the fluorescence intensities at 340 nm and 380 nm. For the $[cAMP]_i$ assays, transfectants cultured in 24-well plates (about 3×10^5 cells/well) were washed with PBS, preincubated for 10 min at 37°C in 0.5 ml of PBS containing 1 mM 3-isobutyl-1-methylxanthine, and exposed to 100 nM S1P in the presence or absence of 10 μM forskolin for 15 min at 37°C. The reactions were terminated by aspirating the medium and adding 75% ice-cold ethanol. The amounts of cAMP extracted from each well were quantified by enzyme-linked immunoassay according to the manufacturer's recommendation.

Measurement of [^{3}H]Inositol Phosphate (IP) Formation. HTC4 cell transfectants expressing each subtype of S1P receptor were incubated overnight in 6-well plates (1×10^6 cells/well). Cells were washed with HEPES-buffered Hank's balanced saline solution, pH 7.4, and incubated for 4 h with 10 μCi of *myo*-[^{3}H]inositol. Cells were then washed and incubated for 10 min with 10 mM LiCl and 1 mM *myo*-inositol. Each incubation was stopped at the indicated times after addition of 10 nM S1P with 5% trichloroacetic acid on ice. Cell lysate supernatants were extracted three times with water-saturated ether and neutralized with 0.2 M Tris buffer, pH 8.0. [^{3}H]IPs were separated by Dowex AG-1X8 columns and measured according to published procedures (Berridge et al., 1983).

To assess the involvement of PTX-sensitive G proteins, some aliquots of cells were incubated for 6 h in the presence of 50 ng/ml of PTX before the determination of S1P-induced changes in $[Ca^{2+}]_i$, IP formation and $[cAMP]_i$.

Results

Most cultured lines of mammalian cells express endogenous S1P receptors, resulting in high background values in measurements of Ca^{2+} mobilization by recombinant receptors using the conventional fluorescence indicator method. Thus, we applied a different method that uses the photoprotein apoaequorin from coelenterate jellyfish *Aequorea victoria*. Apoaequorin forms a bioluminescent aequorin complex with the luminophore coelenterazine. Upon binding to Ca^{2+}, this aequorin complex emits luminescence, which can be detected by luminometry. The cDNA for apoaequorin can be

expressed in mammalian cells, allowing sensitive detection of recombinant GPCR-mediated intracellular Ca^{2+} mobilization (Button and Brownstein, 1993; Brini et al., 1995). When transiently coexpressed with GPCRs in the same subpopulation of cells, aequorin preferentially recorded Ca^{2+} signals mediated by the transfected GPCRs. We took advantage of this sensitive method to record Ca^{2+} mobilization elicited by the cloned S1P receptors with high signal/background ratios.

We chose TAg-Jurkat cells because of their high yields of expression plasmids containing the SV40 replication origin and their relatively low background responses. When used in the fura-2 fluorescence indicator assay, untransfected TAg-Jurkat cells responded to S1P with small but significant increases in $[Ca^{2+}]_i$ (data not shown). Transient transfection of H218, Edg3, or Edg1 did not further elevate the Ca^{2+} responses (data not shown). However, when the aequorin luminescence assay was used, the untransfected or vector-transfected TAg-Jurkat cells showed only minimal responses to S1P. Whereas, cells cotransfected with H218 or Edg3 showed significant increases in light emission resulting from Ca^{2+} binding to aequorin (Fig. 1A). In H218 and Edg3 transfectants, light emission increased immediately after S1P addition, peaked at 9 to 12 s, and then gradually returned to baseline levels within 60 s. The magnitude of S1P-induced light emission in Edg3-transfected cells was consistently greater than that in H218-transfected cells (Fig. 1A). As controls, the vector-transfected TAg-Jurkat T cells were stimulated with monoclonal antibody C305 against the T cell receptor β chain (Fig. 1B). The time course of light emission elicited by C305 showed a longer delay than that evoked by S1P, which is consistent with the slow kinetics of calcium mobilization through T cell receptor activation observed using the fluorescence indicator method. Ionomycin at 1 μM generated a large increase in light emission, which gradually returned to baseline levels in 2 min (Fig. 1B).

In contrast, TAg-Jurkat cells transfected with Edg1 did not show significant S1P-induced increases in luminescence (Fig. 2). To confirm expression of Edg1 in these cells, we employed a chimeric G_q protein, G_{qi5}, which has C-terminal five amino acids changed from $G_{q\alpha}$ to $G_{i\alpha}$ residues allowing G_i-coupled receptors to stimulate PLC (Conklin et al., 1993). When cotransfected with G_{qi5}, Edg1 was able to significantly increase aequorin luminescence in response to 100 nM S1P (Fig. 2). These results suggested that Edg1 was indeed expressed in the TAg-Jurkat cells, and that Edg1 has the potential to mediate Ca^{2+} responses if the G_i-PLC pathway is active in the cells.

S1P increased $[Ca^{2+}]_i$ represented by increases in aequorin luminescence in a concentration-dependent manner (Fig. 3A). The mean EC_{50} values obtained from three independent experiments were 8.0 $\pm$ 3.7 nM and 11 $\pm$ 5 nM for H218 and Edg3, respectively. Two other lysosphingolipids, SPC and dHS1P, also possess biological activities similar to S1P and were shown to interact with H218 and Edg3 in SRE reporter gene transactivation (An et al., 1997). However, in the Ca^{2+} assays, the EC_{50} of SPC and dHS1P were at least two orders of magnitude higher than that of S1P for Edg3 (Fig. 3B) and H218 transfectants (data not shown). The abilities of H218 and Edg3 to recognize several other phospholipids were also examined (Fig. 4). At 1 μM, none of the other sphingolipids and lysophospholipids tested, including SP, dHSP, dMSP, PS, C6-Cer, or LPA induced significant responses in either H218 or Edg3 transfectants (Fig. 4).

To further study the characteristics and mechanisms of Ca^{2+} responses elicited by the recombinant H218 and Edg3 S1P receptors, we established cell lines stably expressing H218 and Edg3. A rat hepatoma cell line, HTC4, was selected after extensive search for a cell line possessing the lowest level of endogenous S1P receptors detectable by Ca^{2+} response measurements and SRE reporter gene assays. We found that untransfected and vector-transfected HTC4 cells

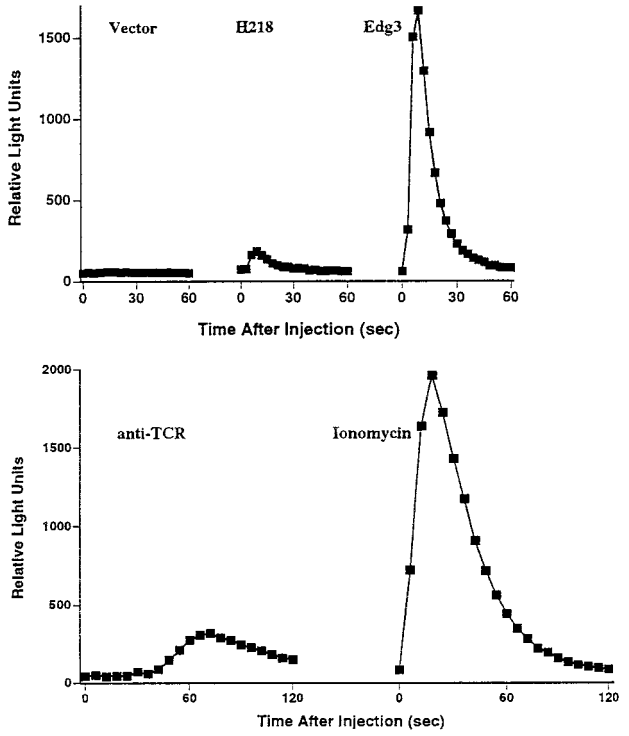

Fig. 1. Kinetics of S1P-elicited aequorin luminescence in H218 and Edg3 transient TAg-Jurkat cell transfectants. TAg-Jurkat T cells were cotransfected by cytAEQ/pcDNA1 with control pCDEF3 vector, H218/EF3, or Edg3/EF3, at a 1:5 ratio, for 10 h. After incubation with 5 μM coelenterazine *f* for 2 h at 37°C, cells were washed, resuspended in PBS, and applied to 96-well plates (2×10^4 cells in 0.1 ml/well). Light emission resulting from Ca^{2+} binding to aequorin was recorded every 3 s with an EG&G Berthold microplate luminometer, after automated injection of indicated ligands. A, light emission in relative light units of vector, H218, or Edg3 transfectants after injection of 10 nM S1P at time 0; B, light emission of control vector transfectants after injection of a 1/1000 dilution of C305 ascites anti-T cell receptor (anti-TCR) or 1 μM ionomycin.

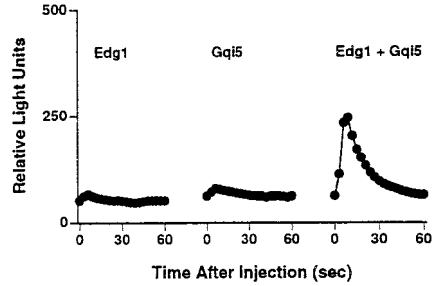

Fig. 2. Edg1-mediated responses in aequorin-Ca^{2+} luminescence in transient TAg-Jurkat cell transfectants requires coexpression of G_{qi5} chimeric G protein. TAg-Jurkat T cells were cotransfected by cytAEQ/pcDNA1 with Edg1/EF3 (left), G_{qi5} (center), or Edg1/EF3 plus G_{qi5} (in 1:1 ratio) (right). Light emission resulting from Ca^{2+} binding to aequorin was recorded every 3 s with an EG&G Berthold microplate luminometer, after automated injection of 100 nM S1P at time 0.

had no detectable background increases in $[Ca^{2+}]_i$ in response to S1P at concentration < 100 nM. In contrast, stably-transfected cell lines expressing H218 or Edg3 showed large increases in $[Ca^{2+}]_i$ in response to S1P. The effects are significant with 0.1 nM S1P and reached a maximum at 100 nM S1P, with EC_{50} around 1 nM (Fig. 5A). SPC and dHS1P generated increases in H218 or Edg3 transfected cell lines at concentrations higher than that of S1P (Fig. 5B), consistent with the rank order of potencies obtained from the aequorin luminescence assay (Fig. 3B). At 100 nM, other structurally similar sphingolipids, such as SP, dHSP, dMSP, PS, and C6-Cer, failed to generate any Ca^{2+} responses (data not shown).

A difference in the kinetic curves for Ca^{2+} flux with H218 and Edg3 was observed (Fig. 6). This difference exists in all clones of each receptor examined despite the broad range of magnitudes of $[Ca^{2+}]_i$ increases, suggesting it is not the result of different levels of receptor expression. For Edg3, the curve maintained a plateau after an initial increase that lasted for >10 min. Whereas for H218, maximal increases in $[Ca^{2+}]_i$ returned to the baseline levels within 2 min after the transient increase (Fig. 6). To determine the sources of Ca^{2+} mobilized by H218 and Edg3, changes in $[Ca^{2+}]_i$ were measured after chelation of extracellular Ca^{2+}. Incubation with EGTA (final concentration, 3 mM) for 1 min before S1P addition reduced the peak heights. More significantly, chelation of extracellular Ca^{2+} eliminated the persistent plateau in Edg3 (Fig. 6), suggesting that the sustained increase for Edg3 was attributable to an influx of extracellular Ca^{2+}. Consistent with these results, pretreatment of cells for 2 min with 20 μM SK&F96365, an inhibitor of receptor-mediated Ca^{2+} entry (Merritt et al., 1990), did not eliminate the initial transient increase in H218 or Edg3, but almost completely eliminated the plateau in Edg3 (Fig. 6). Thus, the sustained

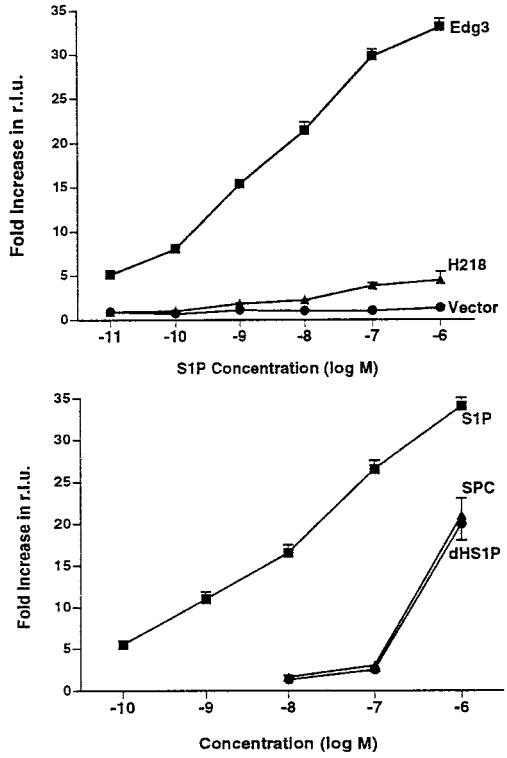

Fig. 3. Concentration dependence of effects of S1P, SPC, and dHS1P on H218- and Edg3-mediated increases in aequorin luminescence. Aequorin light emission of TAg-Jurkat cell transfectants with vector, H218, or Edg3 was recorded for 15 s, beginning immediately after injection of indicated sphingolipids, with a Turner Design 20/20 luminometer. A, various concentrations of S1P from 0.01 to 1000 nM; B, responses of Edg3 transfectants with 0.1 to 1000 nM concentrations of S1P or 10 to 1000 nM concentrations of SPC or dHS1P. Data are expressed as fold increases in relative light units (r.l.u.), and each point is mean ± S.E. of triplicate determinations from one representative experiment of three.

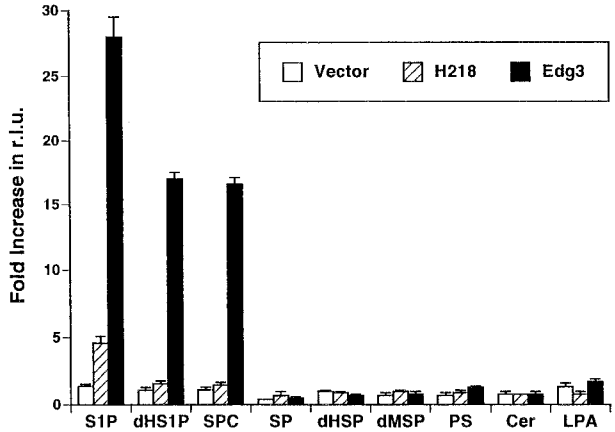

Fig. 4. Effects of S1P, related sphingolipids, and LPA on H218- and Edg3-mediated increases in aequorin luminescence. Aequorin light emission of TAg-Jurkat cell transfectants with vector, H218, or Edg3 was recorded for 15 s, beginning immediately after injection of indicated ligands, with a Turner Design 20/20 luminometer. Concentrations were 100 nM for S1P and 1000 nM for other lipids. Data are expressed as fold increases in relative light units (r.l.u.). Each data point depicts mean ± S.E. of triplicate determinations from one representative experiment of three.

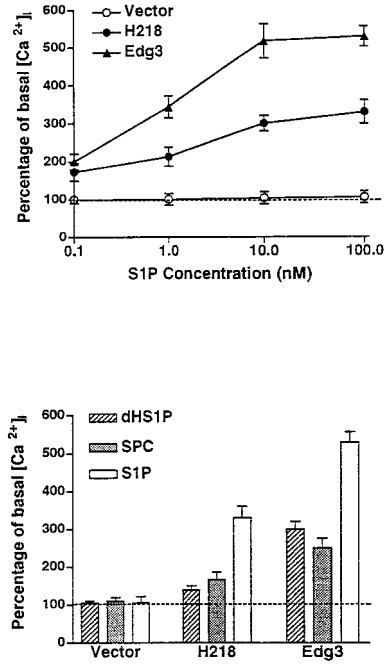

Fig. 5. Effects of S1P and other sphingolipids on $[Ca^{2+}]_i$ measured by fluorescence indicator fura-2 in HTC4 cells stably transfected with vector, H218, or Edg3. Fluorescence of HTC4 transfectants loaded with fura-2 was recorded before and after addition of S1P or other sphingolipids at indicated concentrations. A, dose-dependent responses to S1P at 0.1 to 100 nM; B, dHS1P, SPC, and S1P all at 100 nM. Each data point reported is mean ± S.E. (three experiments) of results of a representative cell line. Values are percentages of basal $[Ca^{2+}]_i$ levels (100%), which were 49 ± 8, 51 ± 6, and 55 ± 8 nM for vector, H218, and Edg3, respectively.

plateau in Edg3 is due to receptor-activated Ca^{2+} influx, and the immediate transient increase in $[Ca^{2+}]_i$ in both receptors is mainly from the release of intracellular sources.

The Ca^{2+} release from intracellular sources was further analyzed by employing thapsigargin (TG), an irreversible inhibitor of the Ca^{2+}-ATPase of ER, that depletes intracellular Ca^{2+} pools (Takemura et al., 1989). The addition of 3 μM TG to EGTA-treated H218 and Edg3 transfectants evoked an increase in $[Ca^{2+}]_i$, which returned to baseline levels in 3 min (Fig. 7). This depletion of internal TG-sensitive Ca^{2+} pool prevented any increase in $[Ca^{2+}]_i$ by subsequent addition of S1P (Fig. 7). In contrast, when S1P was added first, TG still caused a significant, albeit reduced increase in $[Ca^{2+}]_i$ (Fig. 7). These data suggest that S1P-inducible Ca^{2+} pool is entirely contained within the TG-sensitive internal Ca^{2+} stores.

It has been well established that Ca^{2+} mobilization from internal stores elicited by GPCR activation is mediated through IP_3 generated by PLCβ breakdown of phosphatidylinositol phosphates. Previously, it was shown that S1P mobilized Ca^{2+} via G proteins that activate phosphoinositide-specific PLC (Bornfeldt et al., 1995; Okajima et al., 1996; Im et al., 1997; Okajima et al., 1997; Noh et al., 1998). To test whether recombinant H218 and Edg3 mobilize intracellular Ca^{2+} through activation of PLC, we pretreated cells with the U73122 PLC inhibitor before S1P addition. Pretreatment with 3 μM U73122 for 2 min completely blocked S1P-induced increases in $[Ca^{2+}]_i$ in both H218 and Edg3 transfectants (Fig. 8). U73343, an inactive compound used as a control for U73122, did not significantly change the magnitude of Ca^{2+} responses (Fig. 8). To assess the involvement of PTX-sensitive G proteins in S1P-induced Ca^{2+} mobilization by H218 and Edg3, cells were pretreated with 50 ng/ml PTX for 6 h. This PTX treatment partially blocked S1P-induced Ca^{2+} responses in H218 and Edg3 transfectants by 31% ± 5% and 33% ± 8%, respectively (mean ± S.E., n = 6 experiments). As controls, the same PTX treatment almost completely eliminated LPA-induced Ca^{2+} mobilization with Edg2 LPA receptor transfectants of HTC4 cells (An et al., 1998), and completely blocked S1P suppression of adenylyl cyclase in Edg3 transfectants (An et al., unpublished observation).

We next measured the production of radiolabeled total IP and IP_3 after S1P stimulation. Treatment of H218 and Edg3 transfectants with 10 nM S1P for 30 min significantly increased cellular formation of total IP (Fig. 9A). Treatment of H218 and Edg3 transfectants with 10 nM S1P for 1 and 5 min also significantly increased IP_3 production (Fig. 9B). The magnitudes of the increases in both total IP and IP_3 were greater in Edg3 than in H218 transfectants, parallel to the magnitudes of the increases in $[Ca^{2+}]_i$ (Fig. 5).

Sensitivity to inhibition by PTX in S1P-induced IP formation for H218 and Edg3 was also examined (Fig. 9C). PTX treatment partially blocked total IP formation in H218 and Edg3 transfectants by 42% ± 6% and 49% ± 9%, respectively (mean ± S.E., n = 4 samples), but almost completely blocked it in Edg2 transfectants (91% inhibition). As controls, the same PTX treatment did not significantly change IP formation (Fig. 9C) and Ca^{2+} responses (data not shown) induced by 10 μM ATP, which can mobilize intracellular Ca^{2+} predominantly through G_q-coupled purinergic receptors (Parr et al., 1994). The coupling of Edg3 to G_i protein in HTC4 transfectants was further confirmed by the observation that S1P suppressed $[cAMP]_i$ elevated by 10 μM forskolin, and that this suppressive effect of S1P on adenylyl cyclase was eliminated by PTX treatment (An et al., unpublished observation).

Discussion

Although the capacity of S1P to increase $[Ca^{2+}]_i$ in many cell types has been well documented, its signal transductional mechanism remains obscure. The recent identification of Edg1, H218, and Edg3 as receptors for S1P has provided the molecular basis for a GPCR-mediated mechanism (An et

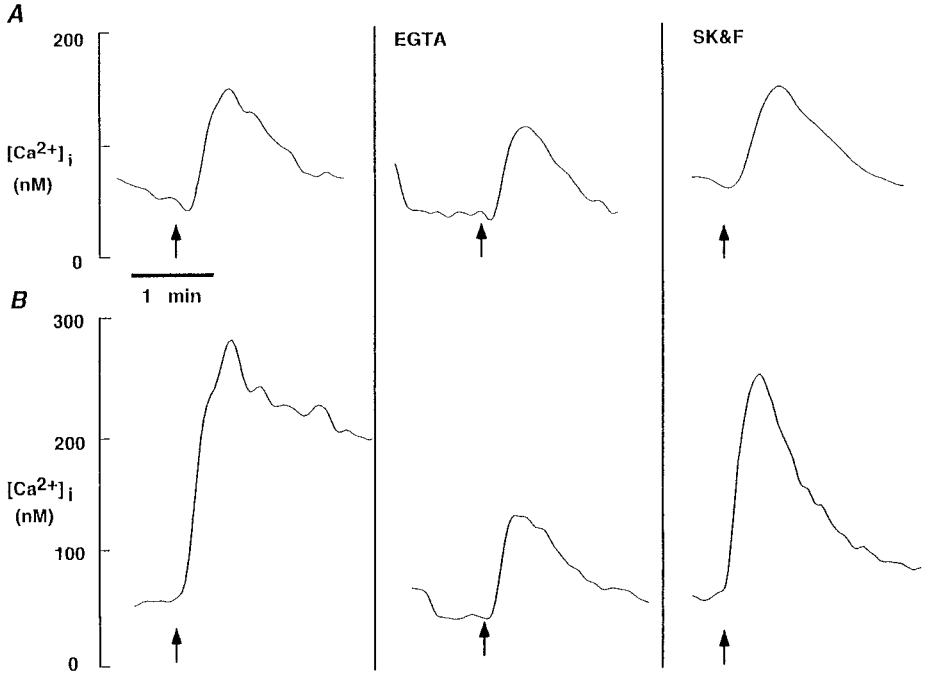

Fig. 6. Measurement of $[Ca^{2+}]_i$ with fluorescence indicator fura-2 in HTC4 cells stably transfected with H218 (A) or Edg3 (B). HTC4 transfectants were loaded with fura-2, and fluorescence was recorded after stimulation with 10 nM S1P (arrows). Left, no EGTA; Center, 3 mM EGTA added 1 min before addition of S1P; Right, 20 μM SK&F96365 was presented 2 min before S1P. Data shown are representative of more than six experiments each with three different stably transfected cell lines for each receptor.

al., 1997; Lee et al., 1998; Zondag et al., 1998). Expression of these receptors in mammalian cells has allowed verification of the GPCR-mediated mechanism and characterization of the signaling properties of S1P-elicited Ca^{2+} responses mediated by each specific subtype.

To overcome difficulties in characterizing cloned S1P receptors resulting from background responses in many cultured mammalian cells, we applied the aequorin luminescence method to monitor increases in $[Ca^{2+}]_i$ mediated by the recombinant S1P receptors. H218 and Edg3 were able to mobilize intracellular Ca^{2+} when expressed in Jurkat cells. These Ca^{2+} responses were mediated specifically by H218 and Edg3, because untransfected and vector-transfected Jurkat cells did not respond to S1P. The EC_{50} of S1P-induced Ca^{2+} responses for H218 and Edg3 is around 10 nM, which is similar to that of native receptors in cultured cells (Okajima et al., 1996; van Koppen et al., 1996; Törnquist et al., 1997), and within the concentration range obtained from biological fluids (Yatomi et al., 1997a). Edg3 transfectants consistently showed greater increases than did H218 transfectants. Because we were unable to quantify the cell surface expression levels of H218 and Edg3 proteins in these Jurkat cell transfectants, it was not possible to compare the intrinsic activities of these two receptors. Nevertheless, we were able to use Ca^{2+} responses as functional readouts to test the relative activities of several natural sphingolipids on H218 and Edg3. We found that the specificities and rank orders of potency of ligands were similar for H218 and Edg3. By far, S1P is the most potent natural ligand of the phospholipids tested. When the aequorin luminescence method was used, the same ligand specificities and rank orders of potency were observed as with the fura-2 fluorescence method (Figs. 3–5). The high

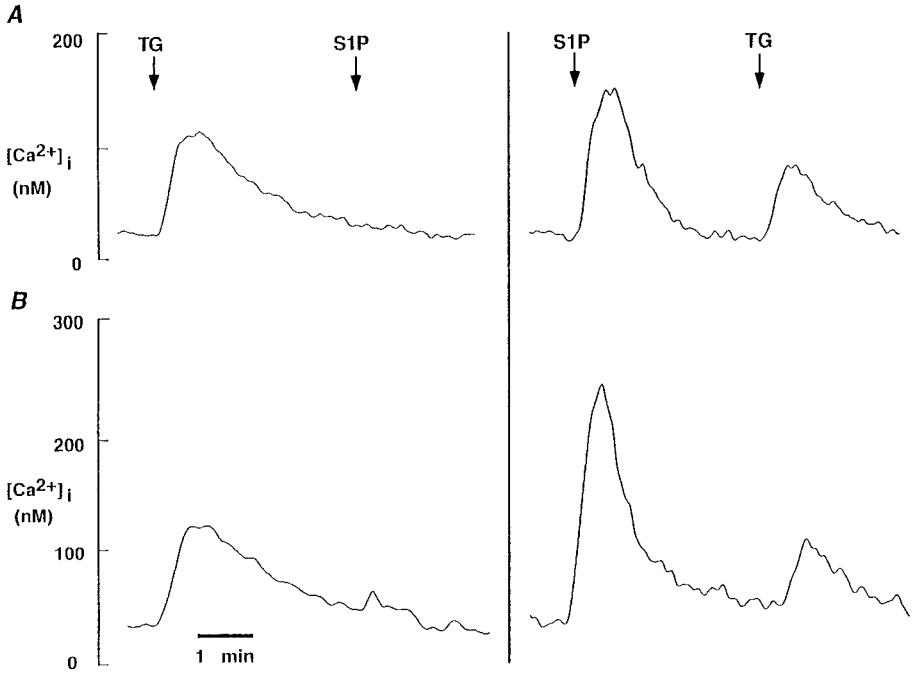

Fig. 7. Effects of TG on S1P-induced increases in $[Ca^{2+}]_i$ in HTC4 cells stably transfected with H218 (A) or Edg3 (B). In presence of 3 mM EGTA, TG (3 μM final concentration) was added before (left) or after (right) addition of 10 nM S1P.

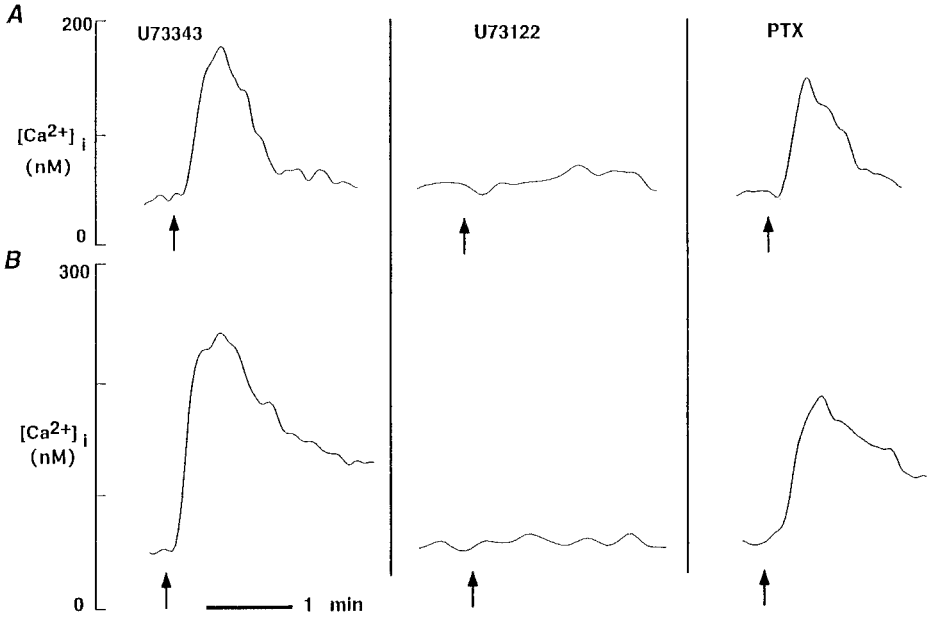

Fig. 8. Effects of U73343, U73122, and PTX on S1P-induced Ca^{2+} mobilization in HTC4 cells stably transfected with H218 (A) or Edg3 (B). HTC4 transfectants loaded with fura-2 were stimulated with 10 nM S1P (arrows), and fluorescence was recorded. U73343 (left), and U73122 (center) were added to final concentrations of 3 μM 2 min before S1P addition. PTX treatment (right) was at 50 ng/ml for 6 h. Data shown are representative of more than six experiments.

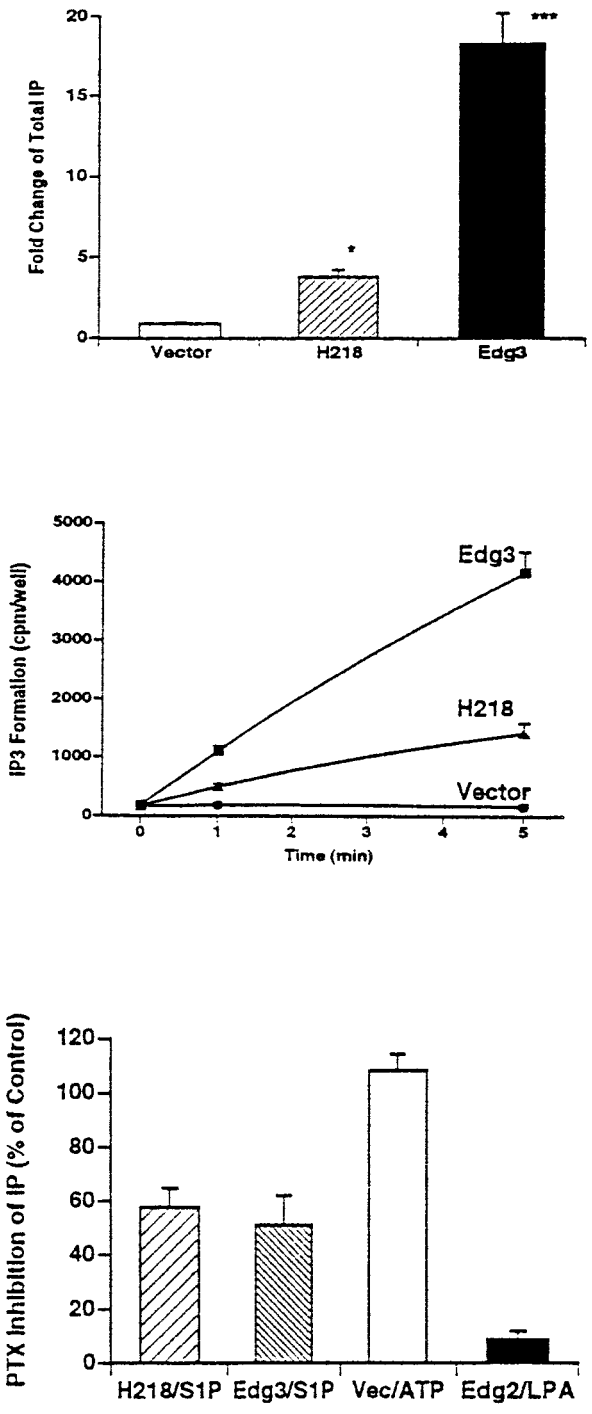

Fig. 9. Effects of S1P on [^{3}H]IP formation in HTC4 cells stably transfected with vector, H218, or Edg3. Each data point depicts mean ± S.E. of quadruplicate samples from one representative experiment of three. A, formation of total IP in 30 min after addition of 10 nM S1P. Basal levels attained before S1P addition were 870 ± 78 cpm for vector, 916 ± 97 cpm for H218, and 1900 ± 160 cpm for Edg3 (mean ± S.E., quadruplicate wells). *$p < .05$; ***$p < .001$ (Student's t test); B, formation of IP_3 before and 1 and 5 min after addition of 10 nM S1P; C, PTX inhibition of total IP formation induced by either 10 nM S1P with H218 or Edg3 transfectants (H218/S1P or Edg3/S1P), 10 μM ATP with vector transfectants (Vec/ATP), or 100 nM LPA with Edg2 LPA receptor transfectants (Edg2/LPA). Data are expressed as percentages of control increments done without PTX (100%). Control increments were 5.4-, 19.8-, 6.0-, and 5.0-fold over unstimulated samples for H218/S1P, Edg3/S1P, Vec/ATP, and Edg2/LPA, respectively.

sensitivity and throughput of the aequorin method may be valuable in large-scale screenings for structure-activity relationships of agonists or antagonists for specific subtypes of S1P receptors.

Several previous studies on Edg1-mediated Ca^{2+} response suggested that Edg1-mediated Ca^{2+} response is cell type-specific. In HEK293 and SF9 cells transfected with Edg1, S1P failed to elicit Ca^{2+} responses (Van Brocklyn et al., 1998; Zondag et al., 1998), whereas in Chinese hamster ovary (CHO) and human erythroleukemia (HEL) cells, Edg1 was able to mediate Ca^{2+} response through $G_{i/o}$ (Okamoto et al., 1998). In the current study with TAg-Jurkat cells, Edg1 was capable of mobilizing Ca^{2+} only when coexpressed with G_{qi5}, which converts G_i-coupled receptors to G_q-activated effectors (Fig. 2). This result, together with previous studies, suggests that Edg1 only couples to G_i, which may or may not elicit a Ca^{2+} response depending on the cellular background. On the contrary, the ability of Edg3 or H218 alone to generate a Ca^{2+} response suggests that they couple to G proteins differently from Edg1 in TAg-Jurkat cells. Mostly likely, Edg3 and H218 couple to G_q proteins to mobilize Ca^{2+} in these cells. This notion is supported by our finding that incubation with 50 ng/ml PTX for 6 h did not significantly change the magnitude of 0.1 μM S1P-induced increases in aequorin luminescence in Edg3- or H218-transfected TAg-Jurkat cells (data not shown).

To further study the characteristics and signaling mechanisms of the Ca^{2+} responses by H218 and Edg3, we established HTC4 cell lines stably expressing H218 and Edg3. A difference in the kinetics of Ca^{2+} flux with H218 and Edg3 was observed. With Edg3, but not with H218, the initial release from TG-sensitive internal stores was followed by a sustained influx of extracellular Ca^{2+} (Fig. 6). It is noted that this sustained plateau in Edg3 was not observed in the aequorin luminescence assay (Fig. 1A). This difference between the two assays might be the result of either the differences between the two assays or cell type-specificity in Edg3-mediated Ca^{2+} flux.

Some studies have proposed an intracellular second messenger role of S1P in Ca^{2+} mobilization that is independent of IP_3 generation. The major lines of evidence that support this mode of S1P action include: first, high concentrations (10–100 μM) of S1P released stored Ca^{2+} directly from the ER; second, heparin antagonism of IP_3 did not block this S1P-induced Ca^{2+} release; and third, inhibitors of sphingosine kinases, dMSP and dHSP, also blocked FcεRI- and muscarinic acetylcholine receptor-mediated Ca^{2+} responses (Ghosh et al., 1994; Mattie et al., 1994; Choi et al., 1996; Meyer zu Heringdorf et al., 1998). To test whether H218- and Edg3-mediated Ca^{2+} mobilization is achieved by generation of S1P intracellularly, we examined whether dMSP, at concentration that reportedly inhibited sphingosine kinases, would alter S1P-induced Ca^{2+} responses. But, dMSP is not a suitable agent to address this issue because dMSP alone at 10 μM induced a sustained elevation of $[Ca^{2+}]_i$ even in vector-transfected HTC4 cells (data not shown). This observation suggests that dMSP may directly alter intracellular Ca^{2+} homeostasis, a notion supported by a recent finding that dMSP at 5 μM blocked Ca^{2+} release-activated Ca^{2+} current (store-operated Ca^{2+} current) by 96% (Mathes et al., 1998). The effects of dMSP other than the inhibition of sphingosine kinases have not been fully characterized, and caution

should be taken when drawing any conclusion about its effects on agonist-induced Ca^{2+} responses.

In summary, we demonstrated that S1P mobilized Ca^{2+} through the defined cell surface GPCRs, H218 and Edg3, with nanomolar EC_{50} values. Edg1 has the potential to mediate Ca^{2+} responses through the G_i-PLC pathway given a proper cellular background. S1P-induced production of IP and elimination of Ca^{2+} responses by the PLC inhibitor U73122 strongly suggested that IP_3 generated by PLC is responsible for H218 and Edg3-mediated Ca^{2+} mobilization. Partial inhibition of Ca^{2+} mobilization and IP formation by PTX implies that H218 and Edg3 transduce PLC-mediated increases in $[Ca^{2+}]_i$ only partially through G_i proteins. Although these results did not rule out a possible role of S1P as an intracellular second messenger in some other settings, they did provide compelling molecular evidence for a GPCR-mediated mechanism of S1P-induced Ca^{2+} responses.

Acknowledgments

We are grateful to Yvonne Kong for technical assistance and Dr. Edward J. Goetzl for his suggestions regarding the manuscript.

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