Comparison of Intrinsic Activities of the Putative Sphingosine 1-Phosphate Receptor Subtypes to Regulate Several Signaling Pathways in Their cDNA-transfected Chinese Hamster Ovary Cells*

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We examined the actions of sphingosine 1-phosphate (SIP) on signaling pathways in Chinese hamster ovary cells transfected with putative SIP receptor subtypes, i.e. Edg-1, AGR16/H218 (Edg-5), and Edg-3. Among these receptor-transfected cells, there was no significant difference in the expressing numbers of the SIP receptors and their affinities to SIP, which were estimated by [³H]SIP binding to the cells. In vector-transfected cells, SIP slightly increased cytosolic Ca²⁺ concentration ([Ca²⁺_i]) in association with inositol phosphate production, reflecting phospholipase C activation; the SIP-induced actions were markedly enhanced in the Edg-3-transfected cells and moderately so in the AGR16-transfected cells. In comparison with vector-transfected cells, the SIP-induced [Ca²⁺_i] increase was also slightly enhanced in the Edg-1-transfected cells. In all cases, the inositol phosphate and Ca²⁺ responses to SIP were partially inhibited by pertussis toxin (PTX). SIP also significantly increased cAMP content in a PTX-insensitive manner in all the transfected cells; the rank order of their intrinsic activity of SIP receptor subtypes was AGR16 > Edg-3 > Edg-1. In the presence of forskolin, however, SIP significantly inhibited cAMP accumulation at a lower concentration (1–100 nM) of SIP in a manner sensitive to PTX in the Edg-1-transfected cells but not in either the Edg-3 or AGR16-transfected cells. As for cell migration activity evaluated by cell number across the filter of blind Boyden chamber, Edg-1 and Edg-3 were equally potent, but AGR16 was ineffective. Thus, SIP receptors may couple to both PTX-sensitive and -insensitive G-proteins, resulting in the selective regulation of the phospholipase C-Ca²⁺ system, adenylyl cyclase-CAMP system, and cell migration activity, according to the receptor subtype.

Sphingosine 1-phosphate (SIP),¹ one of the sphingolipid metabolites, has recently been suggested to affect a variety of cellular processes (1, 2). These cellular responses elicited by SIP have first been ascribed to the intracellular action of the lipid, because SIP accumulated in the cells in response to some kinds of cytokines, and moreover, SIP induced Ca²⁺ mobilization in a cell-free system (3–5). On the other hand, these SIP-induced responses are also accompanied by the stimulation of several early signaling events that are usually regulated by cell-surface receptors. These signaling events include activation of PLC (6–9), an increase in [Ca²⁺_i] (10–12), regulation of adenylyl cyclase (6, 9, 10, 13), and Rho activation (14, 15). The presence of the latter mechanism has been supported by the recent identification of several cDNAs encoding G-protein-coupled receptors for SIP, i.e. Edg-1, AGR16/H218, and Edg-3 (16–23).

The transfection experiments of these SIP receptor subtypes demonstrated that these putative SIP receptors can actually couple to multiple signaling pathways. For example, transfection of Edg-1 induced the inhibition of CAMP accumulation in HEK293 cells (22), S9 cells (18), and Chinese hamster ovary (CHO) cells (20), extracellular signal-regulated kinase activation in COS-1 cells (19), COS-7 cells (18), and CHO cells (20), and activation of Rho, resulting in a morphological change in HEK293 cells (19, 22). The transfection of Edg-1 also caused activation of PLC and [Ca²⁺_i] increase in CHO cells and HEL cells (20), although the overexpression of Edg-1 in HEK293 cells and in SIP cells failed to affect the SIP-induced activation of PLC-Ca²⁺ system (18, 22). The expression of Edg-3 and AGR16 resulted in the activation of a serum response element transcriptional reporter gene in Jurkat cells and the stimulation of Ca²⁺ flux in Xenopus oocytes in response to SIP (17). Coupling of Edg-3 (21) and AGR16 (23) to the Ca²⁺ signaling has recently been confirmed in CHO cells and K562 cells.

Thus, the previous transfection experiments suggest the involvement of these putative SIP receptor subtypes in the regulation of multiple signaling pathways. However, in these experiments, the responses to SIP were observed in the different species of cells, and the receptor numbers expressed in the cells have not always been estimated. Thus, it is difficult to evaluate and compare the intrinsic activity of the respective receptor subtype to regulate a specific signaling pathway from the previous transfection experiments. In the present study, we prepared the CHO cells that permanently express the respective

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¹ The abbreviations used are: SIP, sphingosine 1-phosphate; CHO, Chinese hamster ovary; PTX, pertussis toxin; [Ca²⁺_i], cytoplasmic-free Ca²⁺ concentration; PLC, phospholipase C; G-protein, GTP-binding regulatory protein; IBMX, 3-isobutyl-1-methylxanthine; IP₁, inositol monophosphate; IP₂, inositol bisphosphate; IP₃, inositol trisphosphate; DMEM, Dulbecco’s modified Eagle’s medium; GTPγS, guanosine 5’-3-O-(thio)triphosphate.
S1P receptor subtype to a comparable level. This made it possible to compare their intrinsic activity to regulate the respective signaling pathway. Our data suggested that S1P selectively regulates multiple signaling pathways according to the receptor subtype.

**EXPERIMENTAL PROCEDURES**

**Materials**—1-Oxoeyl-sn-glycero-3-phosphate (lysophosphatic acid) and 3-erythro-sphingosine were purchased from Sigma; sphingosine 1-phosphate (S1P) was purchased from Cayman Chemical Co.; sphingo-1-phosphate (S1P) was purchased from Cayman Chemical Co.; sphingosine kinase-catalyzed phosphorylation as described previously (12). [3H]S1P was separated on silica gel 60 high performance thin-layer chromatography plates (Merck) in a solvent system of butanol-water-acetic acid (3:1:1). By this method, we could obtain [3H]S1P with the same specific activity as the labeled sphingosine. The sources of all other reagents were the same as described previously (21, 24–27).

**Cell Cultures**—CHO cells that had been transfected with the pEFneo expression plasmid were cultured in DMEM containing 10% fetal calf serum (Life Technologies, Inc.) in a humidified air/CO2 (19:1) atmosphere. The cells were described previously (21). Where indicated, the results were normalized to 105 dpm of the total radioactivity incorporated into the cellular inositol lipids. The radioactivity of the trichloroacetic acid (5%) insoluble fraction was measured as the total radioactivity.

**Isolation of cDNAs for S1P Receptors and Construction of Expression Plasmid**—The cDNAs for putative S1P receptors were cloned by reverse transcription-polymerase chain reaction for Edg-1 (29) and AGR16 (30). Using primers that correspond to the N-terminal region of the receptor proteins. The amplified fragments were digested with the restriction enzymes as described above and put in the pBluescript II plasmids (Stratagene), and the DNA sequence was checked.

To construct the S1P receptor expression plasmid, the amplified fragment was inserted into the EcoRI/XhoI site of the pEFneo expression plasmid for Edg-3 and into the HindIII/XhoI site of pEFneo expression plasmid for Edg-1 and AGR16. Of the three types of the putative S1P receptors, the wild type CHO cells expressed the mRNA of AGR16 (30) at 3.1 kilobases but not Edg-3 or Edg-1 (their expected size is 2.8 kilobases for Edg-3 (32) and 3.0 kilobases for Edg-1 (31)). The CHO cells were transfected with pEFneo empty vector alone or the pEFneo vector containing Edg-1, AGR16, or Edg-3, and the neomycin (G418) sulfate at 1 mg/ml-resistant cells were selected. We prepared three batches of CHO cells that were transfected with the empty vector or the respective receptor subtype DNA. Since there was no appreciable difference in the amount of expression of S1P receptor transcript at around 1.8 kilobases between three batches of transfected cells regardless of the receptor subtype, all the data presented in the present study were from one batch of transfected cells. As shown in Fig. 1, there was no stimulatory activity on the membranes of S1P binding to CHO cells among these receptor cDNA-transfected cells.

**Measurement of S1P Receptor Binding**—This was performed by the methods slightly modified from those previously described (19). The cells were washed twice with an ice-cold Tri-s-buffered medium consisting of 20 mM Tri-s-HCl (pH 7.5), 100 mM NaCl, 15 mM NaF, and 0.4% (v/v) bovine serum albumin (fraction V). The medium was replaced with a fresh medium containing [3H]S1P at 3.125 to 100 nM. The plates were kept on ice for 30 min, and the cells were then washed twice with the same ice-cold medium to remove the unbound ligand. The cells were solubilized with a cell-solubilizing solution composed of 0.1% SDS, 0.4% NaOH, and 2% Na2CO3, and the radioactivity was counted. The specific S1P binding to its receptor was estimated by subtracting the radioactivity in the presence of unlabeled S1P.

**Measurement of [3H]Insitol Phosphate Production**—The [3H]inositol-labeled cells were harvested from the 10-cm dishes with trypsin (0.05% in phosphate-buffered saline containing 0.53 mM EDTA) and washed by sedimentation (250 × g × 5 min) and resuspension in the Hepes-buffered medium. The Hepes-buffered medium consisted of 20 mM Hepes (pH 7.5), 134 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 2 mM CaCl2, 2.5 mM NaHCO3, 5 mM glucose, and 0.1% (w/v) bovine serum albumin (fraction V). The washing procedure was repeated, and the cells were finally resuspended in the same medium. Unless otherwise specified, the cells (about 5 × 106 cells) were preincubated for 5 min with 10 mM LiCl in polypropylene vials (20 ml) in a final volume of 2.0 ml. The test agents (×10) were then added to the medium, and the cells were further incubated for the indicated time. The cell suspension (0.5 ml) in triplicate was transferred to tubes containing 1 ml of CHCl3/CH3OH/HCl (100:100:1). [3H]-Labeled respective inositol phosphorus including P1, P2, and IP, were separated as described previously. Where indicated, the results were corrected to 105 dpm of the total radioactivity incorporated into the cellular inositol lipids. The radioactivity of the trichloroacetic acid (5%) insoluble fraction was measured as the total radioactivity.

**Accumulation of cAMP in Intact Cells**—The cells were washed once and preincubated for 10 min at 37 °C in the Hepes-buffered medium. The cells were then incubated with test agents in the presence of 0.5 mM IBMX to estimate the stimulatory activity of adenylyl cyclase (Fig. 6) or in the presence of 0.5 mM IBMX and 10 μM forskolin to estimate the inhibitory activity of adenylyl cyclase (Fig. 5). After a 10-min incubation, the reaction was terminated by adding 100 μl of 1 N HCl. Cyclic AMP in the acid extracts was measured as described previously (9).

**Adenylyl Cyclase Activity in Cell-free System**—Crude membranes were prepared as described previously (28). Briefly, the CHO cells transfected with the respective S1P receptor were harvested from the dishes and washed once with phosphate-buffered saline. The cells were suspended in 50 mM Hepes (pH 7.4) containing 50 mM sucrose, 1 mM EDTA, and 2 μg/ml apronin, then homogenized in a Phosphor hemo-}
indicated concentrations of [3H]S1P. The specific S1P binding was plotted against the S1P concentration. In B–E, Scatchard plots are shown; B/F is plotted against B, in which B is the specific S1P binding (pmol/mg), and F is free (unbounded) S1P concentration (nM). The maximal binding \( B_{\text{max}} \) and the binding dissociation constant \( K_d \) of the respective receptor subtype, i.e. AGR16 (C), Edg-1 (D), and Edg-3 (E) were estimated from the Scatchard plot. These values are shown in the each panel except for vector-transfected cells (B), because of no significant correlation between B and B/F; the correlation coefficient was 0.08, 0.95, 0.86, and 0.93 in the empty vector, AGR16, Edg-1, and Edg-3-transfected cells, respectively.

### RESULTS

**Expression of a Comparable Amount of S1P Receptors in CHO Cells Transfected with their cDNAs.** In A, the vector (○), AGR16 (▲), Edg-1 (●), or Edg-3 (●)-transfected cells were incubated with the indicated concentrations of [3H]S1P. The specific S1P binding was plotted against the S1P concentration. In B–E, Scatchard plots are shown; B/F is plotted against B, in which B is the specific S1P binding (pmol/mg), and F is free (unbounded) S1P concentration (nM). The maximal binding \( B_{\text{max}} \) and the binding dissociation constant \( K_d \) of the respective receptor subtype, i.e. AGR16 (C), Edg-1 (D), and Edg-3 (E) were estimated from the Scatchard plot. These values are shown in the each panel except for vector-transfected cells (B), because of no significant correlation between B and B/F; the correlation coefficient was 0.08, 0.95, 0.86, and 0.93 in the empty vector, AGR16, Edg-1, and Edg-3-transfected cells, respectively.

**Activation of PLC-Ca²⁺ System by Edg-3 and AGR16—Consistent with the previous study (21), the S1P-induced accumulation of inositol phosphate was remarkable in the Edg-3-transfected cells (Figs. 2, left panels and 3D).** The AGR16-transfected cells (Figs. 2, left panels and 3B), but not Edg-1-transfected cells (Figs. 2, left panels and 3C), also displayed a higher ability than the vector-transfected cells to produce inositol phosphate in response to S1P. The difference in the inositol phosphate response is specific to S1P; the response to UTP, a P2 purinergic agonist, was hardly affected by the transfection of any receptor subtype (Fig. 2, right panels). PTX treatment suppressed by 50–80% the S1P action (Fig. 3). Thus, for the intrinsic activity to activate PLC, Edg-3 was the highest followed by AGR16, and a significant effect was not detected by Edg-1.

The activation of PLC is usually accompanied by an increase in [Ca²⁺]. As expected from the inositol phosphate response,
S1P induced a small but significant \([\text{Ca}^{2+}]_i\) increase in the vector-transfected cells, possibly through endogenous S1P receptors (Fig. 4A). The S1P-induced increase in \([\text{Ca}^{2+}]_i\) was markedly enhanced in the Edg-3-transfected cells (Fig. 4D) and moderately so in the AGR16-transfected cells (Fig. 4B). In this case, the S1P-induced \([\text{Ca}^{2+}]_i\) increase was slightly higher in the Edg-1-transfected cells (Fig. 4C) than in the vector-transfected cells (Fig. 4A). The change in the \([\text{Ca}^{2+}]_i\) response was specific to S1P, as evidenced by the observation that the lysophosphatidic acid-induced action was hardly affected by any receptor transfection (Fig. 4E). PTX treatment partially suppressed the S1P-induced actions in all cases.

**Regulation of Adenylyl Cyclase by S1P Receptor Subtypes**—To estimate the activity of adenylyl cyclase in intact cells, the change in cAMP content was measured in the presence of IBMX, a potent inhibitor of phosphodiesterase. In the experiments shown in Fig. 5, forskolin, an activator of adenylyl cyclase, was also supplemented in the incubation medium to evaluate the inhibitory ability of S1P against adenylyl cyclase. In the vector-transfected cells, S1P had no significant effect at less than 100 nM S1P but significantly inhibited it at more than 1 \(\mu M\) (Fig. 5A). This inhibitory action was completely reversed by the treatment of the cells with PTX (Fig. 5A), suggesting the \(G_i/G_o\)-protein-mediated action. When the cells were transfected with the respective receptor subtype, the pattern of the cAMP response to S1P was changed in a manner specific to each receptor. At the concentration lower than 100 nM S1P, where the lipid exerted no detectable effect on forskolin-induced cAMP accumulation in the control vector-transfected cells, S1P slightly enhanced it in the AGR16-transfected cells (Fig. 5B), conversely inhibited it in the Edg-1-transfected cells (Fig. 5C), and exerted no apparent effect in the Edg-3-transfected cells (Fig. 5D). The inhibitory effect of S1P at lower concentration (1–100 nM) in the Edg-1-transfected cells was reversed by PTX treatment (Fig. 5C). This suggests the coupling of Edg-1 to G\(_i\)/G\(_o\)-proteins, resulting in the inhibition of adenylyl cyclase. At concentrations of S1P higher than 1 \(\mu M\), the lipid-induced inhibitory action was apparently attenuated in both cells expressing AGR16 or Edg-3 (Fig. 5, B and D). Interestingly, in
AGR16- and also Edg-3-transfected cells, which were treated with PTX, the cAMP level was significantly increased by increasing the concentration of S1P (Fig. 5, B and D).

We next examined the ability of the respective receptor subtype to stimulate adenylyl cyclase. For this purpose, the experiments were done without forskolin addition (Fig. 6). Under these conditions, S1P hardly changed the cAMP level in vector-transfected cells, but its level significantly increased in response to S1P in all the cells transfected with the receptor subtype. The cAMP level increased around 2 times in the Edg-1-transfected cells (Fig. 6C) and roughly 10 times both in the AGR16- and Edg-3-transfected cells in response to 10 μM S1P (Fig. 6, B and D). However, at lower concentrations of S1P of less than 100 nM, the S1P-induced cAMP accumulation was higher in AGR16-transfected cells than Edg-3-transfected cells (Fig. 6, B and D). PTX did not exert an appreciable effect on the stimulatory action of S1P on cAMP accumulation in all the cases. This striking stimulatory action on the adenylyl cyclase in the cells expressing AGR16 or Edg-3 might partly account for the disappearance of the inhibitory action of S1P (Fig. 5).

It has been reported that adenylyl cyclase activation is in some cases regulated secondarily to the change in Ca\(^{2+}\) signaling (33). As shown in Fig. 7, the PLC inhibitor alone significantly increased the cAMP content by an unidentified mechanism, but the net S1P-induced cAMP accumulation was not appreciably affected by the enzyme inhibitor. Under these conditions, the S1P-induced [Ca\(^{2+}\)]\(_i\) increase was inhibited by more than 80% (21). Thus, it is unlikely that the S1P-induced activation of the PLC-Ca\(^{2+}\) system may be responsible for the lipid-induced cAMP accumulation.

To further provide more direct evidence that the S1P receptor itself is coupled to the adenylyl cyclase system, we measured the enzyme activity in a cell-free system in Fig. 8. Compared with the results in intact cells, S1P effect was small, but the lipid significantly increased adenylyl cyclase activity in the
The enzyme activities (nmol/mg/10 min) in the absence of the guanine nucleotide were 3.22 ± 0.14 and 2.74 ± 0.15 for vector, 2.43 ± 0.04 and 3.65 ± 0.19 for AGR16, 2.42 ± 0.17 and 2.85 ± 0.16 for Edg-1, and 2.56 ± 0.07 and 2.45 ± 0.08 for Edg-3 in the absence and presence of S1P, respectively. Thus, there was no significant effect of S1P in the absence of GTP except for membranes prepared from AGR16-transfected cells, in which a small but significant effect of S1P was observed, possibly due to the endogenous guanine nucleotide. To evaluate the guanine nucleotide-dependent activation of adenyl cyclase, the enzyme activation was induced by 10 nM GTP\(\gamma\)S (the difference in the activity with and without GTP\(\gamma\)S) in the absence (open column) or presence (hatched column) of S1P. * indicates the effect of S1P was significant (p < 0.05).

**FIG. 8.** Stimulation of adenyl cyclase depending on a guanine nucleotide in a cell-free system. The membrane preparations were incubated for 10 min with or without 3 \(\mu\)M S1P and 10 \(\mu\)M GTP\(\gamma\)S. The enzyme activities (nmol/mg/10 min) in the absence of the guanine nucleotide were 3.22 ± 0.14 and 2.74 ± 0.15 for vector, 2.43 ± 0.04 and 3.65 ± 0.19 for AGR16, 2.42 ± 0.17 and 2.85 ± 0.16 for Edg-1, and 2.56 ± 0.07 and 2.45 ± 0.08 for Edg-3 in the absence and presence of S1P, respectively. Thus, there was no significant effect of S1P in the absence of GTP\(\gamma\)S except for membranes prepared from AGR16-transfected cells, in which a small but significant effect of S1P was observed, possibly due to the endogenous guanine nucleotide. To evaluate the guanine nucleotide-dependent activation of adenyl cyclase, the enzyme activation was induced by 10 nM GTP\(\gamma\)S (the difference in the activity with and without GTP\(\gamma\)S) in the absence (open column) or presence (hatched column) of S1P. * indicates the effect of S1P was significant (p < 0.05).

**FIG. 9.** Dose-dependent effect of S1P on cell migration. In A, the vector (●), AGR16 (▲), Edg-1 (△), or Edg-3 (●)-transfected cells were loaded into the upper wells of the Boyden chamber, and cell migration activity for 4 h was measured. The lower wells were filled with the indicated concentrations of S1P. In B (Edg-1) and C (Edg-3), the cells were treated with or without PTX, and then cell migration activity was monitored in the presence (hatched column) or absence (open column) of 10 nM S1P. The number of the cells migrating into the lower surface of the membrane filter (per 0.60-mm\(^2\) field) were counted. Data are means ± S.E. of three separate experiments.

| Effector/response | Receptor subtype |
|------------------|-----------------|
| PLC-Ca\(^{2+}\) stimulation | Edg-3 > AGR16 > Edg-1 |
| Adenylyl cyclase stimulation | AGR16 > Edg-3 > Edg-1 |
| Adenylyl cyclase inhibition | Edg-1 |
| Stimulation of cell migration | Edg-1 = Edg-3 | AGR16 |

**TABLE I** Comparison of intrinsic activities of the S1P receptor subtypes to regulate PLC, adenyl cyclase and cell migration

Discussion

In the present study, we compared the intrinsic activity of the putative S1P receptors, i.e., Edg-1, AGR16, and Edg-3 to induce activation of PLC-Ca\(^{2+}\) system, inhibition of adenylyl cyclase, stimulation of adenylyl cyclase, and stimulation of cell migration activity in CHO cells that permanently express the respective S1P receptor subtype to a comparable level. Results are summarized in Table I.

One of the universal actions of S1P is the activation of PLC and the subsequent increase in [Ca\(^{2+}\)], (6–12), although the involvement of PLC in the [Ca\(^{2+}\)] increase has not always been demonstrated (6, 10–12). Actually, transfection of Edg-1 (20), AGR16 (23), or Edg-3 (21) has been reported to enhance the S1P-induced activation of PLC and/or [Ca\(^{2+}\)] increase. Thus, all the putative S1P receptors have the potential to activate the PLC-Ca\(^{2+}\) system. However, the present study revealed that, in CHO cells expressing an almost equal number of S1P receptors, the intrinsic activity to activate the PLC-Ca\(^{2+}\) system varied in a manner specific to the subtype, i.e., their order being Edg-3 > AGR16 > Edg-1. We detected a small but significant enhancement of the S1P-induced [Ca\(^{2+}\)], increase in the Edg-1-expressing cells (Fig. 4) without a significant effect for the inositol phosphate response (Figs. 2 and 3). The failure of the Edg-1 effect on the inositol phosphate response might simply reflect a lower sensitivity for the detection of the PLC assay compared with the [Ca\(^{2+}\)], measurement. Alternatively, it might indicate the existence of the inositol phosphate-indepen-
dent mechanism for \([Ca^{2+}]_i\) increase. In other cell types, how-
ever, the overexpression of Edg-1 failed to stimulate \(Ca^{2+}\)
signaling (18, 22). Thus, Edg-1 has a potential activity to couple
to the \(Ca^{2+}\) signaling, but its intrinsic activity is very small
compared with Edg-3 or AGR16, and hence this receptor sub-
type might not be important for the regulation of the \(Ca^{2+}\)
signaling in the native cells.

We have recently found that only Edg-3 mRNA expression
was detected among three types of S1P receptors in undiffer-
entiated HL-60 cells, and its expression was down-regulated
in association with the attenuation of the S1P-induced \(Ca^{2+}\)
response during differentiation of the cells (37). This suggests
that Edg-3 may couple to the PLC-\(Ca^{2+}\) system in HL-60 cells.
In the native CHO cells, mRNA expression of AGR16, but
neither Edg-1 or Edg-3, was detected (27). Similarly, in FRTL-5
cells, we detected only AGR16 mRNA expression (data not
shown). In these cells, S1P seemed to increase \([Ca^{2+}]_i\), depend-
ing on PLC activation (9, 21). Thus, both Edg-3 and AGR16
may be involved in the S1P-induced activation of the PLC-\(Ca^{2+}\)
system in the native cells as well. S1P-induced PLC activation
and \([Ca^{2+}]_i\) increase have, in most cases, been reported to be
attenuated by PTX treatment in native cells (6, 7, 10, 11).
Similarly, the S1P-induced actions in the present study were
also partially suppressed by PTX treatment (Figs. 3 and 4).
Although the complete ADP-ribosylation of \(G_{i/o}\)-proteins was
not proven by the PTX treatment employed in the present
study, the inhibitory action of S1P on the forskolin-induced
cAMP accumulation was completely reversed (Fig. 5). This
suggests that the function of \(G_{i/o}\)-proteins may be lost under
these conditions. On the other hand, the stimulatory action
of S1P on cAMP accumulation was unaltered by the toxin
activation (Fig. 6), excluding the possible nonspecific toxic action
of PTX. Thus, Edg-3 and AGR16 may couple to both PTX-sensi-
tive \(G_{i/o}\)-proteins and probably, the toxin-insensitive \(G_{q/11}\)-
proteins, resulting in the activation of PLC and the subsequent
\(Ca^{2+}\) mobilization from the intracellular pool.

Consistent with the previous results (18, 20, 22), Edg-1 may
couple to the inhibitory adenylyl cyclase system through PTX-
sensitive \(G_{i/o}\)-proteins (Fig. 5). On the other hand, we could
not detect the inhibitory action of S1P in either the Edg-3 or
the AGR16-expressing cells, in which S1P elicited a rather stimulatory action on cAMP accumulation (Fig. 6). However,
the cAMP level was significantly higher in PTX-treated cells at
lower concentrations of less than 100 nM S1P (Fig. 5, B and D),
suggesting that the inhibitory S1P action was masked by the
stimulatory S1P action in the cells not treated with PTX. In
relation to this, it should be noted that in the native CHO cells,
only AGR16 mRNA expression was detected among three types
of the putative S1P receptor (27). Therefore, the inhibition
of cAMP accumulation by higher concentrations (more than 1
\(\mu M\)) of S1P, which was observed in the vector-transfected cells (Fig. 5), might be mediated by the endogenous AGR16. Further experi-
ments are necessary to define conclusively the ability of Edg-3
and AGR16 to couple to the inhibitory adenylyl cyclase system.

In addition to the inhibitory action on cAMP accumulation,
S1P has in some cases been reported to stimulate cAMP accumu-
lation reflecting activation of adenylyl cyclase, especially in
smooth muscle cells (13). The order of the intrinsic activity of
the stimulatory action on cAMP accumulation in the present
receptor subtype-transfected cell system was AGR16 > Edg-3 > Edg-1. It is unlikely that the activation of adenylyl cyclase
was the secondary action of the lipid-induced activation of
PLC-\(Ca^{2+}\) system (Fig. 7). Actually, at least a part of the cAMP
response may reflect the lipid receptor/\(G_{i/o}\)-protein-mediated ac-
tivation of adenylyl cyclase as evidenced by the guanine nucleo-
tide-dependent activation of the enzyme by S1P in a cell-free system
(Fig. 8). However, we cannot completely rule out the possibility
that S1P activated the enzyme partly through the production of
autocrine stimulators such as prostaglandin in intact cells.

Finally, we evaluated the ability of the respective S1P recep-
tor subtype to migrate the cells. Transfection of Edg-1 or Edg-3
into the CHO cells stimulated cell migration in response to
S1P, whereas neither the vector nor AGR16 transfection ap-
preciately affected the migration activity. Although the signal-
ing mechanisms involved in the cell migration have not been
well characterized, disassembly and assembly of actin filament
may be involved in this process. The receptor-mediated rearr-
angement of actin filament has been shown to involve the
\(G_{12/13}\) family of G-proteins and the Rho family of small G-
proteins (14, 15, 38, 39). In the present study, the S1P-induced
action was markedly suppressed by PTX treatment. In this
case, the basal activity was also suppressed by the toxin treat-
ment (Fig. 9), suggesting that \(G_{i/o}\)-proteins might be abso-
lutely necessary for the induction of cell migration. However,
this result never rules out the possible involvement of \(G_{12/13}\)
proteins in the cell migration. Thus, both \(G_{i/o}\)-proteins and
\(G_{12/13}\) proteins might cooperatively regulate the cell migra-
tion activity. In the previous study, S1P has been shown to
stimulate migration of T-lymphoma (15) similar to the S1P
receptor-transfected CHO cells but conversely to inhibit migra-
tion of smooth muscle cells, neutrophils, and melanoma cells (2, 13).
This cell type-dependent discrepancy in S1P effects on cell
migration is intriguing. The difference in the expression of the
S1P receptor subtype might be responsible for the opposite
direction of the response to S1P. This deserves further inves-
tigation in a future study.

At the present stage of investigation, AGR16 and Edg-1 have
been identified from rat and AGR16, Edg-1, and Edg-3 from
human. In the present study, we used rat AGR16, rat Edg-1,
and human Edg-3. Therefore, it is not completely excluded that
the difference in the intrinsic activity of the receptor subtype
to regulate the several signaling pathways might in part reflect
the differences in the animal species. However, the amino acid
sequence is very similar between rat and human; the homology
is 92 and 90% overall in the coding region of Edg-1 and AGR16,
respectively (16, 29, 30). In a future study, the intrinsic activity
should be compared within the receptors from the same animal
species. In this way, we could know the portion or domain
within the receptor that is important for the regulation of the
respective signaling pathway by transfection experiments us-
ing the chimera and mutated receptors.

Last but not least, in the present study each S1P receptor
subtype was expressed in the CHO cells that were expressing
many types of cell surface receptors including one of the S1P-
receptor, AGR16. The interaction or synergism between more
than two receptors is one mode of signaling mechanism for
some extracellular ligands including ATP and adenosine (40,
41). Thus, there might be the interaction between the trans-
fected receptors and the endogenous S1P receptors, which
might result in the modification of the intrinsic activity of the
transfected receptor, although such a cross-talk mechanism
has not yet been reported in the case of the S1P receptors.
Therefore, to evaluate a more accurate intrinsic activity of the
respective S1P receptor subtype for the regulation of a specific
signaling pathway, similar experiments may be necessary in
the cells that are not expressing endogenous S1P receptors in a
future study.

In conclusion, the putative S1P receptors that have recently
been identified may couple to the PLC-\(Ca^{2+}\) system, adenylyl
cyclase-cAMP system, and actin rearrangement-cell motility
system through PTX-sensitive and -insensitive G-proteins in a
manner selective to their subtype.
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