# Activation of phospholipase C-$Ca^{2+}$ system by sphingosine 1-phosphate in CHO cells transfected with Edg-3, a putative lipid receptor

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Received 14 December 1998

**Abstract Sphingosine 1-phosphate (S1P) induces phospholipase C (PLC) activation and $Ca^{2+}$ mobilization in many types of cells. We examined the possible involvement of Edg-3, one of the putative S1P receptors, in the phospholipase C (PLC)-$Ca^{2+}$ system. S1P increased the cytoplasmic free $Ca^{2+}$ concentration without detectable inositol phosphate production in vector-transfected CHO cells. In the Edg-3-transfected cells, however, the S1P-induced $Ca^{2+}$ response was clearly enhanced, which was associated with a significant production of inositol phosphate. These S1P-induced responses in the Edg-3-transfected cells were inhibited by U73122, a potent PLC inhibitor. We conclude that Edg-3 may be one of the S1P receptors participating in the activation of the PLC-$Ca^{2+}$ system.**



*Key words:* Sphingosine 1-phosphate; Edg-3; $Ca^{2+}$; Phospholipase C; CHO cell

## 1. Introduction

Sphingosine 1-phosphate (S1P), one of the sphingolipid metabolites, has recently been suggested to be involved in the regulation of a variety of cellular processes [1,2]. There are at least two mechanisms with respect to the action mode for exogenous S1P, i.e. intracellular mechanisms through intracellular targets molecules (which have not yet been identified) and extracellular mechanisms through cell surface receptors [1,2]. The presence of the latter mechanism has been supported by the recent identification of several cDNAs encoding G-protein-coupled receptors for S1P, i.e. Edg-1, AGR16/H218 and Edg-3 [3–5].

As for the S1P-induced $Ca^{2+}$ mobilization, two action mechanisms have also been reported. S1P was first reported to directly act on the internal $Ca^{2+}$ pool resulting in $Ca^{2+}$ mobilization in a way similar to inositol 1,4,5-trisphosphate in permeabilized cells or in the purified endoplasmic reticulum [6–8]. S1P is accumulated in response to PDGF and serum in Swiss 3T3 fibroblasts; hence, this lipid has been proposed as a second messenger of PDGF and serum during cell proliferation in Swiss 3T3 fibroblasts [9]. A second messenger role of S1P has also been suggested for the IgE receptor [10] and some G-protein-coupled receptors [11]. In HL-60 cells, we have shown that exogenous S1P also increased $Ca^{2+}$ but this $Ca^{2+}$ increase was associated with phospholipase C (PLC) activation [12]. These S1P-induced actions were markedly suppressed by PTX, suggesting an involvement of PTX-sensitive G-proteins in the lipid signaling. Furthermore, these lipid actions were also inhibited by the dibutyryl cAMP-induced differentiation of the cells, in which the downstream signaling pathway from the G-proteins was rather fortified [12,13]. This ruled out the possibility of G-proteins as the action site of S1P, and hence we proposed that the S1P-induced activation of the PLC-$Ca^{2+}$ system is mediated through the G-protein-coupled receptors in HL-60 cells [12]. Participation of the G-proteins or cell surface receptors in the S1P-induced $Ca^{2+}$ mobilization has also been suggested in other cell types including fibroblasts [14], HEK 293 [15], endothelial cells [16], platelets [17], hepatocytes [18] and thyroid cells [19], although the involvement of PLC has not always been demonstrated for the $[Ca^{2+}]_i$ increase [14–16]. However, the receptor subtype involved in activation of the PLC-$Ca^{2+}$ system has not yet been identified.

We have recently found that only Edg-3 mRNA expression was detected among the three putative lipid receptors in HL-60 leukemia cells and this mRNA expression was markedly attenuated in association with the inhibition of the S1P-induced $Ca^{2+}$ response during differentiation induced by several agents including dibutyryl cAMP [20]. This raised the possibility that Edg-3 might be a S1P receptor linking to the $Ca^{2+}$ signaling. To confirm this, in the present study, we transfected Edg-3 cDNA into CHO cells which express AGR16/H218 but do not express detectable amounts of Edg-3 and Edg-1 mRNAs [21]. The Edg-3-transfected CHO cells markedly activated the PLC-$Ca^{2+}$ system in response to S1P. Thus, Edg-3 may be one of the S1P receptors involved in the activation of the PLC-$Ca^{2+}$ system observed in many types of cells.

## 2. Materials and methods

### 2.1. Materials

1-Oleoyl-*sn*-glycero-3-phosphate (lysophosphatidic acid; LPA) and D-*erythro*-sphingosine were purchased from Sigma; S1P from Cayman Chemical Co.; sphingosylphosphorylcholine (SPC) from Biomol Research Lab. Inc.; Fura 2/AM from Dojindo (Tokyo); and *myo*-[2-$^3$H]inositol (23.0 Ci/mmol) from DuPont-New England Nuclear. U73122 and U73343 were generously provided by the Upjohn Co. (Kalamazoo, MI). The sources of all other reagents were as previously described [12,13,18,19].

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*Abbreviations:* S1P, sphingosine 1-phosphate; G-protein, GTP-binding regulatory protein; PTX, pertussis toxin; $[Ca^{2+}]_i$, cytoplasmic free $Ca^{2+}$ concentration; PLC, phospholipase C; SPC, sphingosylphosphorylcholine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $IP_1$, inositol monophosphate; $IP_2$, inositol bisphosphate; $IP_3$, inositol trisphosphate


PII: S0014-5793(98)01676-7

*2.2. Cell cultures*

Chinese hamster ovary (CHO) cells, which were transfected with the pEFneo vector [22] (control cells) or pEFneoEdg-3 vector (Edg-3-transfected cells), were cultured in DMEM containing 10% FBS on 10-cm dishes for $Ca^{2+}$ response and on 6-multiwell plates for inositol phosphate response unless otherwise specified. Twenty-four hours before the experiments, the medium was changed to fresh DMEM (without serum) containing 0.1% BSA. For the $[Ca^{2+}]_i$ measurement, the cells were harvested and suspended cells were used. In the case of the inositol phosphate response, the medium was changed to the inositol-free DMEM containing 2 μCi [$^3$H]inositol (in 1.5 ml) and 0.1% BSA. After 24 h, inositol phosphate response was performed without cell harvest. In the experiments shown in Fig. 4F, however, the cells were labeled on 10-cm dishes in the inositol-free DMEM containing 20 μCi [$^3$H]inositol in 7 ml and the inositol phosphate response was performed in a cell suspension as was case for the $Ca^{2+}$ response. PTX treatment of the cells was performed by adding the toxin (50 ng/ml) to the medium 24 h before the experiments.

*2.3. Measurement of [$^3$H]inositol phosphate production*

In the case of the cells attached to the 6-multiwell plates, the [$^3$H]inositol-labeled cells were washed twice with HEPES-buffered medium which consisted of 20 mM HEPES (pH 7.5), 134 mM NaCl, 4.7 mM KCl, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 2.5 mM $NaHCO_3$, 5 mM glucose, and 0.1% (w/v) bovine serum albumin (fraction V), then preincubated with 0.9 ml of the same medium containing 10 mM LiCl. After 5 min, test agents (×10) in 0.1 ml were added to the medium and further incubated for the indicated time. The reaction was terminated by adding 1 N HCl (0.1 ml). The other procedures were exactly the same as those previously described [12,23]. In the case of the cell suspension (Fig. 4F, lower panel) where the effects of U73122, a PLC inhibitor, and U73343, an inactive derivative, were examined, the [$^3$H]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 with the HEPES-buffered medium. The washing procedure was repeated and the cells were finally resuspended in the same medium. The cells (about $3\times10^6$) were preincubated for 2 min with 5 μM U73122 or 5 μM U73343 in the presence of 10 mM LiCl in polypropylene vials (20 ml) in a final volume of 2.0 ml. The test agents (×100) were then added to the medium and the cells were further incubated for 1 min. The cell suspension (0.5 ml) in triplicate was transferred to tubes containing 1 ml of $CHCl_3$/MeOH/HCl (100/100/1). Where indicated, the results were normalized to $10^5$ 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.

*2.4. Measurement of $[Ca^{2+}]_i$*

This was performed by the fluorescence change of Fura 2-loaded cells. The cells were harvested from the dishes with trypsin as described above. After the 20-min incubation of the cells with 1 mM Fura 2/AM at 37°C, the cells were washed two times with ice-cold HEPES-buffered medium and finally suspended in the same medium. The Fura 2-loaded cells were kept on ice while waiting for $[Ca^{2+}]_i$ measurement. The cells were warmed at 37°C for 1 min and then the $[Ca^{2+}]_i$ change was monitored. If the cells were maintained at room temperature, a higher response to test agents was usually observed compared with the cells which were kept on ice, but we noticed that the basal $[Ca^{2+}]_i$ sometimes increased spontaneously (net increase was about 100 nM) then returned to the initial level (within about 3 min) by stirring the cells, especially in the case of Edg-3-transfected cells. When such a spontaneous $[Ca^{2+}]_i$ increase occurred, the $Ca^{2+}$ response to test agents was markedly suppressed even when $[Ca^{2+}]_i$ was measured after it returned to the initial level. Other experimental conditions are the same as those previously described [23].

*2.5. Isolation of cDNAs for S1P receptors and construction of expression plasmid*

The cDNA of Edg-3 [24] was cloned by RT-PCR from the total RNA of HEK 293 cells with 5′-ggggaattcCCACCATGGCAACTG-CCCTCCCGCCGCG-3′ and 5′-gggtctagaTCAGTTGCAGAAGAT-CCCATTCTG-3′. The 5′ primers contain a restriction enzyme site (*Eco*RI) and a Kozak sequence (CCACC) before the N-terminal region of the receptor proteins. The 3′ primers contain a restriction enzyme site (*Xba*I) and a stop codon in addition to the C-terminal region of the receptor proteins. The amplified fragments were digested with the restriction enzymes, put in the pBluescript II plasmids (Stratagene) and the DNA sequence was checked.

To construct the Edg-3 expression plasmid, the amplified fragment was inserted into the *Eco*RI/*Xba*I site of the pEFneo expression plasmid. Of the three types of the putative S1P receptors that have recently been identified, the wild type CHO cells abundantly express the mRNA of AGR16/H218 [25,26] at 3.1 kb, but not Edg-3 or Edg-1 (their expected size is 2.8 kb for Edg-3 [24] and 3.0 kb for Edg-1 [27]). The CHO cells were transfected with pEFneo vector (control cells) or pEFneo containing Edg-3 (Edg-3-transfected cells) and neomycin (G418 sulfate)-resistant cells were selected. We prepared three sets of transfected cells. These three sets of Edg-3-transfected cells all expressed an Edg-3 transcript around 1.8 kb, although we did not measured the expression of the S1P-receptor proteins. All the data presented in this study were from one set of transfected cells; we observed almost the same results using other sets of cells stably transfected with Edg-3.

*2.6. Data presentation*

All experiments were performed in duplicate or triplicate. The results of multiple observations are presented as the representative or means ± S.E.M. of at least three separate experiments unless otherwise stated.

## 3. Results

Fig. 1 shows the effects of S1P and UTP, a $P_2$-purinergic agonist, on the inositol phosphate production in the control (pEFneo vector-transfected) CHO cells and Edg-3-transfected cells. In the control cells (Fig. 1A–C), S1P failed to accumulate any inositol phosphate species. In the case of UTP, which has been shown to activate PLC in many types of cells, we only detected a significant accumulation of $IP_1$, but not $IP_2$ or $IP_3$. The presence of many species of $IP_2$ and $IP_3$ molecules and their different metabolic rate might mask the significant accumulation of these polyinositol phosphates. In the Edg-3-transfected cells as well (Fig. 1D–F), UTP stimulated only $IP_1$ accumulation to an extent similar to that in the control cells. On the other hand, S1P significantly stimulated all the species of inositol phosphates in the Edg-3-transfected cells. However, the extent of the increase in rate of $IP_2$ and $IP_3$ production (Fig. 1E,F) was less than that of $IP_1$ production (Fig. 1D) at all times employed in these cells, e.g. the percent increase over basal at 1 min after S1P addition was 300% for $IP_1$, 100% for $IP_2$ and only 20% for $IP_3$.

The S1P-induced inositol phosphate production was partially suppressed by PTX treatment, whereas the toxin hardly affected the UTP-induced action; the toxin inhibited about 40% and 20% of the responses as induced by 10 nM S1P and 1 mM S1P, respectively (Fig. 2). In CHO cells, LPA can inhibit forskolin-induced cAMP accumulation in a PTX-sensitive manner; cAMP accumulation (nmol/mg protein) induced by forskolin (10 μM) plus IBMX (0.5 mM) in the absence and presence of LPA (10 μM) was 28.8 ± 0.8 and 20.9 ± 0.5 in the control cells non-treated with PTX and 29.4 ± 1.0 and 31.6 ± 0.8 in the toxin-treated cells, respectively, in the case of Edg-3-transfected cells ($n = 3$). There was no significant difference in the LPA-induced effect and the toxin sensitivity between vector-transfected and Edg-3-transfected cells (data not shown). Thus, PTX-sensitive G-proteins seem to be almost completely inactivated by the toxin treatment employed in the present study.

The dose-dependent effect of S1P and its related compounds on inositol phosphate production is shown in Fig. 3. In vector-transfected cells, even 10 μM S1P was ineffective.

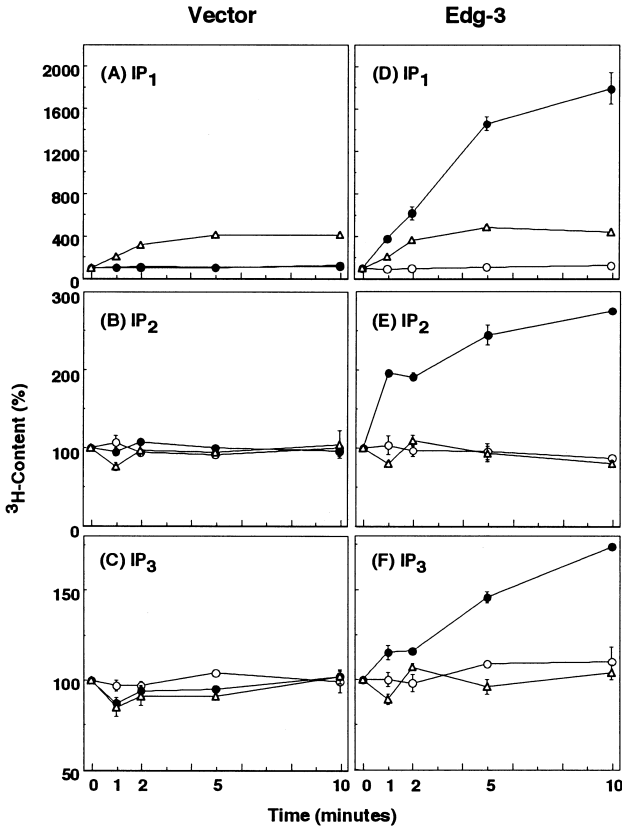


Fig. 1. Effects of S1P and UTP on the individual inositol phosphate production in the control vector-transfected cells (left panels) and in the Edg-3-transfected cells (right panels). The [$^3$H]inositol-labeled cells were incubated for the indicated time with buffer alone (○), 1 μM S1P (●) or 100 μM UTP (△). The production of $IP_1$ (A,D), $IP_2$ (B,E) and $IP_3$ (C,F) was measured. Results are expressed as percentages of the initial values before the addition of the test agents. Normalized initial values (dpm) were 1313 ± 54 and 1180 ± 107 for $IP_1$, 1952 ± 108 and 1643 ± 96 for $IP_2$ and 6085 ± 214 and 5780 ± 362 for $IP_3$ in the control and Edg-3-treated cells, respectively. Data are means ± S.E.M. of three separate experiments.

Similarly, LPA (Fig. 3A), SPC and sphingosine (data not shown) were ineffective for the induction of the inositol phosphate response. LPA was still ineffective in the Edg-3-transfected cells, but both SPC and sphingosine at 10 μM exerted significant effects. Thus, SPC and sphingosine in addition to S1P may interact with Edg-3 to activate PLC, although their potency was about two orders less than that of S1P.

We finally examined whether the inositol phosphate response was actually accompanied by an increase in $Ca^{2+}$ response. In vector-transfected cells, S1P slightly but significantly induced a transient $[Ca^{2+}]_i$ increase and this $Ca^{2+}$ response was completely inhibited by U73122, a PLC inhibitor (Fig. 4A,E), although S1P failed to increase inositol phosphate production in these cells (Figs. 1 and 3). The $Ca^{2+}$ response to S1P was clearly enhanced in the Edg-3-transfected cells (Fig. 4B,D). The enhancement of the $Ca^{2+}$ response was specific to S1P; the LPA-induced action was unchanged by the Edg-3 transfection (Fig. 4C,D). The S1P-induced $[Ca^{2+}]_i$ increase in the Edg-3-transfected cells may also be dependent on PLC activation based on the observation that U73122, a potent PLC inhibitor, clearly inhibited the S1P-induced increase in $[Ca^{2+}]_i$ (Fig. 4B,F) in association with

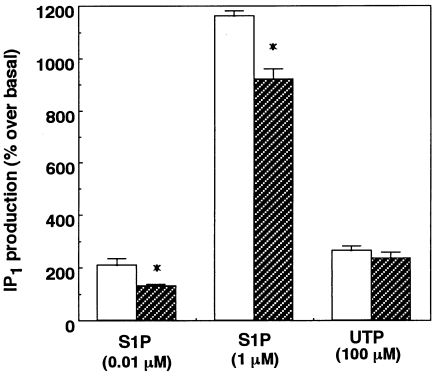


Fig. 2. Effect of PTX on the inositol phosphate production induced by S1P and UTP. The Edg-3-transfected cells labeled with [$^3$H]inositol were incubated for 10 min with the indicated doses of S1P or UTP; cells non-treated with PTX (open column) and PTX-treated cells (hatched column). Production of $IP_1$ was measured, because the UTP effect was only observed in this fraction (see Fig. 1). Results are expressed as percentages over basal values obtained without test agents. Normalized basal values were 1157 ± 54 for cells non-treated with PTX and 1138 ± 77 for toxin-treated cells. *$P < 0.05$, significant from control cells.

an inhibition of the lipid-induced inositol phosphate production (Fig. 4F).

As expected from the PTX effect on inositol phosphate response to S1P in Edg-3-transfected cells (Fig. 2), the toxin significantly inhibited the S1P-induced $[Ca^{2+}]_i$ increase (Fig. 4D). However, this does not simply mean the involvement of PTX-sensitive G-proteins in the Edg-3-mediated $[Ca^{2+}]_i$ increase, because the PTX-sensitive S1P-induced $[Ca^{2+}]_i$ increase was also detected in the vector-transfected cells (Fig. 4C). To estimate the Edg-3-mediated response, we calculated

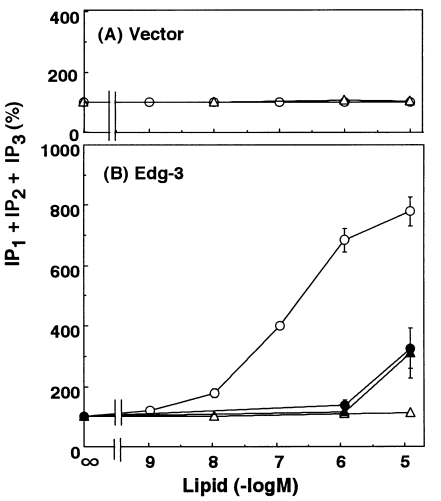


Fig. 3. Effects of S1P and its related compounds on the inositol phosphate production in the control vector-transfected cells (A) and in the Edg-3-transfected cells (B). The [$^3$H]inositol-labeled cells were incubated for 10 min with the indicated doses of S1P (○), LPA (△), SPC (●) or sphingosine (▲). The production of total inositol phosphates ($IP_1$+$IP_2$+$IP_3$) was measured. Results are expressed as percentages of the basal values obtained without the test agents. Normalized basal values (dpm) were 4561 ± 59 and 4706 ± 74 in the control cells and Edg-3-treated cells, respectively. Data are means ± S.E.M. of three separate experiments.

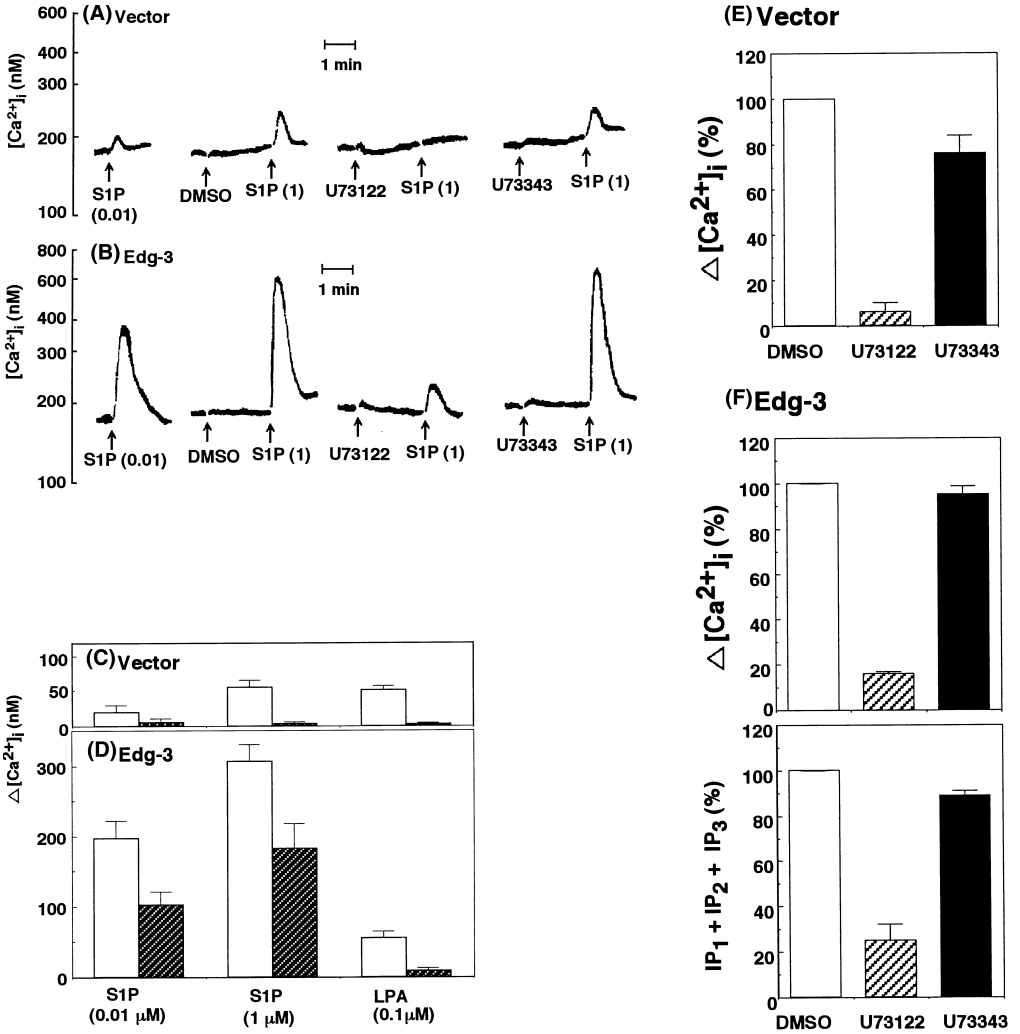


Fig. 4. Effect of Edg-3 transfection on S1P-induced $[Ca^{2+}]_i$ increase and a possible involvement of PLC activation in the $Ca^{2+}$ response. A,B: Representative traces of $[Ca^{2+}]_i$ change by S1P in the control vector-transfected cells (A) and in the Edg-3-transfected cells (B) are shown. At the arrow, S1P (μM), U73122 (5 μM), U73343 (5 μM), or its vehicle (DMSO) was added. C,D: Effects of PTX on the S1P- and LPA-induced $[Ca^{2+}]_i$ increase in the control vector-transfected cells (C) and in the Edg-3-transfected cells (D) are shown. The cells non-treated with PTX (open column) or PTX-treated cells (hatched column) were incubated with the indicated doses of S1P or LPA to monitor the $[Ca^{2+}]_i$ change. The net $[Ca^{2+}]_i$ change (peak value minus basal value) at around 15 s is shown. Data are means ± S.E.M. of eight separate experiments. Effects of PTX were significant in all cases. E,F: Effects of U73122 (hatched column) and U73343 (closed column) on S1P-induced $[Ca^{2+}]_i$ increase and inositol phosphate production are shown. Inhibitor experiments on inositol phosphate response were performed only in Edg-3-transfected cells, since a significant S1P effect was not obtained in vector-transfected cells (see Fig. 1). Similar to the experiments shown in A and B, the cells were first preincubated for 2 min with U73122 (5 μM), U73343 (5 μM) or their vehicle (DMSO) and then S1P (1 μM) was added to the incubation medium. The net $[Ca^{2+}]_i$ changes induced by S1P in the control vector-transfected cells (E) and in the Edg-3-transfected cells (F, upper panel) were measured and are expressed as percentages of values obtained in the control cells (DMSO) taken as 100%. The 100% value was 74 ± 10 nM (E) and 348 ± 35 nM (F). In the lower panel of F, the [$^3$H]inositol-labeled Edg-3-transfected cells were incubated for 1 min with or without 1 μM S1P in the presence of the PLC inhibitor or its inactive derivative in the suspension similarly to the $Ca^{2+}$ response as described in Section 2. The production of total inositol phosphates ($IP_1+IP_2+IP_3$) was measured. The normalized value (dpm) was 1309 ± 36 without S1P and 5006 ± 362 with S1P in the control cells (DMSO). Since the basal values without S1P were not significantly changed by the PLC inhibitor and its inactive derivative, the results are expressed as percentages of the increments induced by S1P in the control cells (DMSO) taken as 100%.

the difference in the respective values between the vector-transfected cells and the Edg-3-transfected cells; these values for non-treated cells and PTX-treated cells are 177 nM and 97 nM at 10 nM S1P and 252 nM and 179 nM at 1 μM S1P, respectively. Thus, PTX treatment inhibited the Edg-3-mediated $Ca^{2+}$ response about 45% at 10 nM S1P and 30% at 1 μM S1P; these values are roughly comparable to the inhibition rate of the Edg-3-mediated PLC activation by PTX (Fig. 2). Thus, the S1P-induced $[Ca^{2+}]_i$ increase may be mediated partly by PTX-sensitive G-proteins as was the case for the lipid-induced PLC activation.

## 4. Discussion

Three types of cDNAs encoding G-protein-coupled receptors for S1P, i.e. Edg-1, AGR16/H218 and Edg-3, have re-

cently been identified by several groups [3–5]. Edg-1 has been suggested to couple to PTX-sensitive G-proteins to inhibit adenylyl cyclase and ERK activation and couple to PTX-insensitive G-proteins to modulate Rho-mediated changes in morphology and motility [4,5,28]. However, its receptor subtype does not seem to be responsible for the $Ca^{2+}$ signaling [4,5,28]. AGR16/H218 and Edg-3 have been shown to stimulate the $Ca^{2+}$ flux in *Xenopus* oocytes [3]. However, the mechanism by which S1P stimulated the $Ca^{2+}$ efflux has not yet been characterized. Furthermore, the observation in *Xenopus* oocytes is not always reproducible in mammalian cell systems. For example, an overexpression of a putative lysophosphatidic acid receptor, PSP24 [29], which was originally cloned from *Xenopus* oocytes, enhanced the lipid-induced $Ca^{2+}$-mediated $Cl^-$ current in the same oocytes, but its action has not yet been confirmed in mammalian cell systems. In the present study, we stably transfected Edg-3 cDNA into CHO cells which do not express detectable Edg-3 mRNA and demonstrated that transfection of Edg-3 actually activated PLC in response to S1P and enhanced the lipid-induced $[Ca^{2+}]_i$ increase in the mammal cells.

The S1P-induced PLC activation may be responsible for the lipid-induced $[Ca^{2+}]_i$ increase in the Edg-3-transfected cells as evidenced from the inhibition of both responses to S1P by U73122, a potent PLC inhibitor. The PTX treatment partially inhibited S1P-induced PLC activation in association with the parallel inhibition of the lipid-induced $Ca^{2+}$ response, further suggesting the participation of PLC in the lipid-induced $[Ca^{2+}]_i$ increase. The inhibition of the S1P-induced actions by PTX also suggests the involvement of PTX-sensitive $G_i/G_o$ proteins in lipid signaling. In the present study, although we did not prove the complete ADP-ribosylation of $G_i/G_o$ proteins by PTX, LPA-induced inhibition of cAMP accumulation and increase in $[Ca^{2+}]_i$ were almost completely suppressed by the toxin treatment, suggesting that the toxin treatment under the present experimental conditions seems to almost completely inactivate the function of $G_i/G_o$ proteins. Taking it into consideration that Edg-3 is a G-protein-coupled type of receptor, the PTX-insensitive S1P-induced PLC activation may be mediated through $G_{11}/G_q$ proteins. In relation to the inhibitory action of LPA on adenylyl cyclase, we preliminarily examined S1P effects on cAMP accumulation. S1P also slightly inhibited the forskolin-induced cAMP accumulation in control CHO cells, but the inhibitory S1P action disappeared in the Edg-3-transfected cells (data not shown). The apparent reversal of the S1P action may be due to the stimulatory action of S1P on cAMP accumulation in the Edg-3-transfected cells. The detailed mechanism of action of S1P on the regulation of cAMP metabolism is our current subject of study. In any event, Edg-3 may couple to several types of G-proteins, i.e. $G_i/G_o$, $G_{11}/G_q$ and $G_s$ proteins, resulting in the stimulation of several intracellular signaling pathways.

In the vector-transfected CHO cells as well, S1P significantly increased $[Ca^{2+}]_i$. This may reflect the abundant expression of AGR16/H218 in the control CHO cells [21]. As already mentioned, AGR16/H218 in addition to Edg-3 has been suggested to stimulate $Ca^{2+}$ flux at least in *Xenopus* oocytes [3]. However, we could not detect any significant increase in the production of inositol phosphates as a reflection of the PLC activation in response to S1P in the control (vector-transfected) cells. The lack of significant production of inositol phosphate may simply reflect the lower sensitivity for the detection of the PLC assay compared with $[Ca^{2+}]_i$ measurement, because the S1P-induced $Ca^{2+}$ response was lost in the presence of a PLC inhibitor (Fig. 4A,E). Alternatively, it might reflect the difference in the mechanism of $Ca^{2+}$ signaling [11] between AGR16/H218 and Edg-3. This problem should be clarified in a future study.

In conclusion, Edg-3, which has been reported to be expressed in many types of tissues and organs including heart, muscle and liver [20,24], may be one of the putative S1P receptors mediating the activation of the PLC-$Ca^{2+}$ system.

*Acknowledgements:* We wish to thank Ms. Yoko Shimoda for her dedicated help during the preparation of the manuscript. This work was supported by a research grant from the Ministry of Education, Science and Culture of Japan and by a research grant from Taisho Pharmaceuticals.

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