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Possible Involvement of Cell Surface Receptors in Sphingosine 1-Phosphate-Induced Activation of Extracellular Signal-Regulated Kinase in C6 Glioma Cells

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ABSTRACT

The early signaling mechanism of sphingosine 1-phosphate (S1P) on extracellular signal-regulated kinase (ERK) activation was investigated in C6 glioma cells. S1P activated the enzyme in association with a shift in the mobility on electrophoresis reflecting phosphorylation of both ERK1/ERK2 at as low as 10 nM. The lipid-induced ERK1/2 activation was partially inhibited by treatment of the cells with either phorbol 12-myristate 13-acetate (a long-term treatment to desensitize protein kinase C) or pertussis toxin (PTX) and was completely inhibited by a simultaneous treatment with both agents. Similarly, either calphostin C, an inhibitor of protein kinase C, or U73122, an inhibitor of phospholipase C, partially inhibited the S1P-induced ERK1/2 activation in the nontreated cells with PTX and completely in the toxin-treated cells. On the other hand, the S1P-induced ERK activation was hardly affected by ethanol, which switched the product of phospholipase D from phosphatidic acid to metabolism-resistant phosphatidylethanol. S1P was able to activate ERK1/2 without a detectable increase in the intracellular content of the lipid, but sphingosine, a substrate of sphingosine kinase, which is an enzyme for S1P generation in the cells, hardly affected the ERK1/2 activation in spite of a marked elevation of intracellular S1P accumulation. This indicates that intracellular increase in S1P is not necessary for the S1P-induced ERK activation, and hence suggests the extracellular action mechanism of S1P. Supporting this idea, mRNAs of recently identified S1P specific receptors, Edg-1 and AGR16/H218, were expressed in C6 cells. Taken together, these results suggested that S1P acts on C6 cells extracellularly possibly through S1P receptors which are linked to at least two signaling pathways, i.e., the PTX-sensitive G_i/G_o protein pathway and the toxin-insensitive G_q/G_{11}-phospholipase C-PKC pathway, resulting in the activation of ERK.

Sphingosine 1-phosphate (S1P), a novel lipid mediator, has recently been suggested to be involved in the regulation of a variety of cellular processes, i.e., cell proliferation (Olivera and Spiegel, 1993; Goodemote et al., 1995; Wu et al., 1995; Pyne et al., 1996; Rani et al., 1997; Blakesley et al., 1997), metabolic regulation (Okajima et al., 1997; Im et al., 1997), morphological changes (Yatomi et al., 1995a; Postma et al., 1996; Yatomi et al., 1997; Sato et al., 1997), and cell motility (Sadahira et al., 1992; Igarashi, 1997). S1P has been demonstrated to be generated from sphingosine (Sph) by sphingosine kinase in response to platelet-derived growth factor (PDGF) in 3T3 fibroblasts (Olivera and Spiegel, 1993). This lysosphingolipid has been reported to directly act on the internal Ca^{++} pool, resulting in Ca^{++} mobilization in a manner similar to that of inositol 1,4,5-trisphosphate (Ghosh et al., 1994; Mattie et al., 1994). Furthermore, PDGF-induced extracellular signal-regulated kinase (ERK) activation, which is suggested to be an important early signaling event for the proliferation, was partially inhibited by DL-*threo*-dihydrosphingosine, a nonselective sphingosine kinase inhibitor, suggesting that a part of PDGF action on ERK activation is mediated by sphingosine kinase-derived S1P in 3T3 fibroblasts (Rani et al., 1997). Actually, exogenously applied S1P activated ERK and cell proliferation regardless of the presence of the sphingosine kinase inhibitor in the same cells (Olivera and Spiegel, 1993; Wu et al., 1995; Rani et al., 1997). Thus, the lipid has been proposed as a second messenger of PDGF on Ca^{++} mobilization and ERK activation, leading to

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ABBREVIATIONS: S1P, sphingosine 1-phosphate; PDGF, platelet-derived growth factor; ERK, extracellular signal-regulated kinase; G-protein, GTP-binding regulatory protein; PTX, pertussis toxin; Sph, sphingosine; CHO, Chinese hamster ovary; PA, 1, 2-diacyl-sn-glycero-3-phosphate; PEt, 1-palmitoyl-2-oleoyl-sn-3-phosphoethanol; PMA, phorbol 12-myristate 13-acetate; Fura-2/AM, [1-[2-5-Carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*′,*N*′,-tetraacetic acid]/acetoxymethyl ester; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PKC, protein kinase C.

cell proliferation in fibroblasts (Olivera and Spiegel, 1993; Wu et al., 1995; Spiegel and Milstien, 1995; Rani et al., 1997). A similar second messenger role of S1P has been proposed for the IgE receptor-mediated $[Ca^{++}]_i$ increase in basophils (Choi et al., 1996).

An alternative mode of signaling for S1P is the activation of extracellular receptors. S1P is known to be released by platelets in response to physiological receptor agonists such as thrombin (Yatomi et al., 1995a). It has been shown that S1P regulated early signaling events including activation of phospholipase C (Goodemote et al., 1995; Okajima et al., 1996; Okajima et al., 1997; Im et al., 1997), increase in $[Ca^{++}]_i$ (Mattie et al., 1994; Okajima et al., 1996; van Koppen et al., 1996; Im et al., 1997; Okajima et al., 1997; Yatomi et al., 1997), inhibition of adenylyl cyclase (Goodemote et al., 1995; van Koppen et al., 1996; Im et al., 1997; Okajima et al., 1997), and activation of the K^+ channel (Bünemann et al., 1996; van Koppen et al., 1996). Most of these S1P-induced actions were suppressed by pertussis toxin (PTX) treatment, suggesting that PTX-sensitive G proteins seem to be involved in the lipid actions (Goodemote et al., 1995; Okajima et al., 1996; van Koppen et al., 1996; Im et al., 1997). In these cases, PTX-sensitive G proteins do not appear to be primary action sites for the lipid (Okajima et al., 1996; van Koppen et al., 1996). Even when the S1P-induced actions were insensitive to PTX, e.g., axon retraction (Postma et al., 1996; Sato et al., 1997) and inhibition of cell motility (Sadahira et al., 1992), injection of S1P into the cells failed to mimic lipid actions (Postma et al., 1996), but nonpermeable S1P effectively mimicked these actions (Sato et al., 1997). These results suggested that S1P acts on the cells extracellularly, possibly through cell surface receptors which are coupled to G proteins. Very recently, a couple of cDNAs encoding G-protein-coupled receptors for S1P have been identified by several groups (An et al., 1997; Lee et al., 1998; Zondag et al., 1998).

The change in ERK activity is accompanied by a variety of cellular events, e.g., cell proliferation, metabolism, secretion, and gene regulation (Gutkind, 1998). Thus, the regulation of ERK is one of the important intracellular signaling events to regulate the cellular activities in response to extracellular signals. S1P also regulated ERK activity in several types of the cells including smooth muscle cells (Pyne et al., 1996) and osteoblasts (Kozawa et al., 1997) in addition to 3T3 fibroblasts (Wu et al., 1995; Blakesley et al., 1997; Rani et al., 1997). However, the signaling mechanisms including primary action sites of the S1P-induced enzyme activation have not been fully elucidated. As mentioned above, when the cells are exposed to S1P, the lipid has the potential to act on the cells both intracellularly and extracellularly. To provide further insight into the mechanisms underlying the S1P actions, in the present study, we analyzed the ERK pathways of C6 glioma cells. In this cell line, we have recently observed that S1P stimulated the induction of fibroblast growth factor 2, one of the potent growth factors for astrocytes and neuronal cells, and Egr-1, an important transcriptional factor for fibroblast growth factor 2 expression; these events were associated with ERK activation (K. Sato, M. Ui, and F. Okajima, in preparation). We found that S1P-induced ERK activation is mediated by at least two signaling pathways, i.e., PTX-sensitive and -insensitive pathways. In the latter toxin-insensitive pathway, the phospholipase C-protein kinase C (PKC) system plays an essential role. Exogenous S1P activated ERK without a detectable increase in intracellular accumulation of S1P, and Sph was ineffective in activating it in spite of a marked accumulation of intracellular S1P accumulation. These results suggested that S1P acts on the cells extracellularly through cell surface receptors which are in part coupled to PTX-sensitive G_i/G_o proteins and in part to the toxin-insensitive G_q/G_{11} proteins. Supporting this idea, mRNAs of Edg-1 and AGR16/H218, which have recently been identified as S1P receptors linked to ERK and Ca^{++}-signaling pathways, were expressed in this cell line.

Experimental Procedures

Materials and Methods

Materials. Sphingosylphosphorylcholine, Sph, phorbol 12-myristate 13-acetate (PMA), calphostin C, and 1,2-diacyl-*sn*-glycero-3-phosphate (L-α-phosphatidic acid, or PA) were purchased from Sigma Chemical Co. (St. Louis, MO); HEPES and Fura-2/acetoxymethyl ester (Fura-2/AM) were purchased from Dojindo (Tokyo, Japan); 1-palmitoyl-2-oleoyl-sn-3-phosphoethanol (phosphatidylethanol or PEt) was obtained from BIOMOL Research Labs., Inc. (Plymouth Meeting, PA); RO 20-1724 was obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA); [^{3}H]acetic anhydride (50 mCi/mmol) and [^{3}H]oleic acid (10 Ci/mmol) were purchased from DuPont-New England Nuclear (Boston, MA); [α-^{32}P]dATP (3000 Ci/mmol), [γ-32P]ATP (3,000 Ci/mmol) and a p44/p42 mitogen-activated protein kinase (ERK 1/2) enzyme assay kit were purchaced from Amersham Corp. (Uppsala, Sweden); and ERK-specific antibody (K-23, amino acids 305–327 of rat ERK 1 which recognizes both ERK 1 and ERK 2) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). U73122 and U73343 were generously provided by Upjohn Co. (Kalamazoo, MI); and a cyclic AMP (cAMP) assay kit was obtained from Yamasa Shoyu Co. (Choshi, Chiba, Japan). S1P was prepared by treatment of sphingosylphosphorylcholine with phospholipase D as described (Okajima et al., 1996). The sources of all other reagents were described previously (Okajima et al., 1988; Okajima and Kondo, 1995; Okajima et al., 1996; Im et al., 1997; Okajima et al., 1997; Sato et al., 1997).

Cell Culture. C6 glioma cells, a rat glioma cell line, obtained from the Japanese Science Research Resources Bank (JCRB9096) were grown in Ham's F10 medium supplemented with heat-inactivated 15% horse serum and 2.5% fetal bovine serum. The cells were plated on 12-well plates for the cAMP measurement, on 6-well plates for the estimation of phospholipase D activity, and on 10-cm plates for other experiments including extraction of total RNA, measurement of ERK1/2 activity, and measurement of $[Ca^{++}]_i$. When the cells had become 70 to 80% confluent, the culture medium was changed to fresh Ham's F10 medium containing 0.1% bovine serum albumin (BSA) to render the cells quiescent for 2 days. PTX and/or PMA treatment of the cells was performed by adding 100 ng/ml PTX and/or 1 μM PMA to the serum-free medium 24 h before the experiments. For the experiment to measure phospholipase D activity, the medium was supplemented with [^{3}H]oleic acid (2 mCi/ml) during the serum-deprivation period for 24 h. The Chinese hamster ovary (CHO) cells, human embryonic kidney 293 cells, and Swiss 3T3 fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Measurement of ERK1/2 Activity. The cells were washed once, preincubated for 10 min at 37°C in a HEPES-buffered medium composed of 20 mM HEPES, pH7.4, 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% BSA (fraction V), and then incubated with test agents for various lengths of time at 37°C. The incubation was terminated by washing twice with ice-cold phosphate-buffered saline (PBS) and adding 0.5 ml of a lysis buffer composed of 50 mM HEPES, pH 7.0, 250 mM NaCl, 0.1% Nonidet P-40, 100 mM NaF, 0.2 mM

sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin, and the cells were then harvested from the dishes with a rubber policeman. The recovered lysate was incubated for 30 min on ice and was centrifuged at 14,000*g* for 20 min. The kinase activity was determined with an assay kit (Amersham Corp.) that measures the incorporation of [γ-^{32}P]ATP into a synthetic peptide (KRELVEPLTPAGEAPNQALLR) as a specific substrate. The protein concentration of the supernatant was determined with the BCA protein assay (Pierce Chemical Co., Rockford IL).

Western Blot Analysis. The same supernatant as that used for ERK activity was also analyzed by Western blotting with an ERK-specific antibody. Protein extracts were subjected to 10% SDS-polyacrylamide gel electrophoresis and proteins in the gel were transferred to a polyvinylidene difluoride membrane (ProBlott; Applied Biosystems, Foster City, CA) by electroblot. The membranes were blocked with 5% dry milk for 2 h and incubated with primary antibodies (1:1,000 dilution) for 2 h. The membranes were then incubated with a second antibody conjugated with alkaline phosphatase for 1 h, and were visualized using the nitro blue teterazolium/5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt system. Prestained molecular weight markers were used to determine the molecular weights of the transferred proteins.

Measurement of Intracellular cAMP Content. The cells were washed once and preincubated for 10 min at 37°C in the HEPES-buffered medium. The medium was replaced with the same medium (0.5 ml) containing 10 μM forskolin, 100 μM RO 20-1724, 0.5 U/ml adenosine deaminase, and various agonists to be tested. After a 5-min incubation, the reaction was terminated by adding 100 μl of 1 N HCl. cAMP in the acid extracts was measured by a sensitive and specific radioimmunoassay as described previously (Im et al., 1997).

Measurement of $[Ca^{++}]_i$. The cells were washed twice with Ca^{++}- and Mg^{++}-free PBS containing 1 mM EGTA, warmed in the same medium for 5 min at 37°C, and gently harvested from dishes with a rubber policeman. The cells were then sedimented by centrifugation at 250*g*, suspended in Ham's F10 medium containing 0.1% BSA, and incubated with 1 μM Fura-2/AM for 20 min at 37°C. The cells were washed twice to eliminate excess Fura-2/AM by repeating the sedimentation at 250*g* and resuspended in the HEPES-buffered medium. The change in $[Ca^{++}]_i$ was estimated from the change in the fluorescence of the Fura-2-loaded cells as described previously (Okajima et al., 1988).

Estimation of Phospholipase D Activity. This was performed by measurement of the production of [^{3}H] PA or [^{3}H]PEt from [^{3}H]-oleic acid-labeled cells (El-Moatassim and Dubyak, 1992). The cells prelabeled with [^{3}H]oleic acid (2 μCi/ml) for 24 h were washed twice and preincubated for 10 min at 37°C in the HEPES-buffered medium. The reaction was started by adding 1.5 ml of the HEPES-buffered medium containing agonists in the presence or absence of 2% ethanol. After 2.5 min, incubations were stopped by aspirating off the medium and subsequently adding 1 ml of 0.2 N HCl solution. The cells were harvested from dishes with a rubber policeman and vigorously mixed with 4 ml of chloroform/methanol (2:1). The lower chloroform phase was collected, dried by vacuum centrifugation, and then dissolved in 15 μl of chloroform/methanol (2:1). The reaction product, PA or PEt, was resolved on Silica Gel 60 thin-layer chromatography plates (Merck; Rahway, NJ) in a solvent system of ethyl acetate/iso-octane/acetic acid (9:5:2). The plates were treated with EN3HANCE Spray (DuPont-New England Nuclear) and autoradiographed by exposure to Kodak X-Omat AR film (Eastman Kodak Co., Rochester, NY) at −80°C using an intensifying screen. The radioactive spots corresponding to PA or PEt were scraped off and counted by a liquid scintillation counter.

Measurement of Intracellular S1P Content. The cells were washed twice and warmed in the HEPES-buffered medium for 10 min at 37°C. The cells were then incubated for 2.5 min with the indicated doses of lipids. The incubation was terminated by washing twice with ice-cold PBS and adding 0.25 ml of 0.1 N HCl, and the cells were then harvested from the dishes with a rubber policeman. The cellular S1P was extracted and measured by acetylation with [^{3}H]acetic anhydride as described (Yatomi et al., 1995b; Im et al., 1997; Sato et al., 1997). The reaction product, *N*-acetylated S1P, was resolved on Silica Gel 60 high-performance thin-layer chromatography plates (Merck) in a solvent system of chloroform/methanol/acetic acid/water (65:43:1:3). The plates were treated with EN3HANCE Spray and autoradiographed as described above. The radioactive spots corresponding to N-acetylated S1P were scraped off and counted by a liquid scintillation counter. The amount of S1P was calculated by extrapolation from [^{3}H]*N*-acetylated S1P standards.

Isolation of cDNAs for S1P Receptors. The cDNAs for putative S1P receptors were cloned by reverse transcription-polymerase chain reaction as follows: Edg-1 (Hla and Maciag, 1990) (1152 bp) from the total RNA of rat brain with 5′-gggaagcttCCACCATGGTGTCCTCCAC-CAGCATCCC-3′ and 5′-gggtctagaTTAAGAAGAAGAATTGACGTT-TCC-3′, AGR16/H218 (Okazaki et al., 1993) (1059 bp) from the total RNA of rat brain with 5′-gggaagcttCCACCATGGGCGGTTTAT-ACTCAGAGT-3′ and 5′-gggtctagaTCAGACCACTGTGTTGCCCTC-3′, and Edg-3 (Yamaguchi et al., 1996) (1137 bp) from the total RNA of HEK 293 cells with 5′-ggggaattcCCACCATGGCAACTGCCCTCC-CGCCGCG-3′ and 5′-gggtctagaTCAGTTGCAGAAGATCCCATTCTG-3′. The 5′ primers contain a restriction enzyme site (*Hin*dIII or *Eco*RI) and a Kozak sequence (CCACC) before the N-terminal region of 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 as described above, put in the pBluescript II plasmids (Stratagene Inc., La Jolla, CA), and the DNA sequence was checked.

RNA Extraction and Northern Blot Analysis. Total RNA isolates were prepared from C6 cells, CHO cells and 3T3 fibroblasts according to the manufacturer's instructions for TRIZOL Reagent (Life Technologies, Inc., Grand Island, NY). Northern blot analysis was performed as described previously (Sato and Di Lauro, 1996). Briefly, 10 μg of total RNA was electrophoresed through a 1% agarose gel containing 3.7% formaldehyde and 20 mM morpholinepropane sulfonic acid buffer and then blotted onto a nylon membrane (Hybond-N; Amersham Corp.) with 20 × standard saline citrate. The probes were labeled with [α-^{32}P]dATP by random oligonucleotide priming. The procedures for hybridization were carried out at 65°C. Following hybridization, the blots were washed with 0.2 × standard saline citrate and 0.1% SDS at 65°C. To normalize the amounts of RNA present in the blots, GAPDH was used.

Data Presentation. All experiments were performed in duplicate or triplicate. The results of multiple observations are presented as the mean ± S.E. or as representative results from more than three different batches of cells unless otherwise stated.

Results

Exogenous S1P Activates ERK Through Two Signaling Pathways. Figure 1A shows a time course of ERK activity change by S1P and Sph. S1P activated the enzyme transiently; its activity peaked at 2.5 min and returned to the basal level at 7 min. In the case of Sph, however, we failed to detect any significant activation during the experimental period (~30 min). The enzyme activation by S1P was associated with a shift in the mobility of both ERK1/ERK2 on a SDS-polyacrylamide gel electrophoresis gel, probably reflecting phosphorylation of the enzymes (Fig. 1B).

PMA was as effective as S1P in activating ERK, suggesting that PKC activation plays a role in the enzyme activation (Fig. 2A). To determine whether PKC is involved in the S1P action, the effect of a long-term treatment of the cells with PMA was examined. After a 24-h treatment of the cells with PMA, the PMA no longer activated the enzyme (Fig. 2A),

demonstrating that PKC is desensitized. Under these conditions, the S1P effect on ERK was markedly suppressed, especially at lower doses of the lipid; however, S1P was still effective at higher doses (Fig. 2C). To further confirm the involvement of PKC in the S1P signaling, we used calphostin C, a potent PKC inhibitor. This drug treatment also markedly but not completely inhibited the S1P action (Fig. 2D). Thus, the S1P-induced ERK activation seems to involve not only PKC-dependent but also enzyme-independent mechanisms.

We next examined PTX effect on the S1P-induced ERK activation. The toxin significantly but not completely inhibited the S1P-induced activation of ERK (Fig. 2C). Although we did not directly prove the complete ADP-ribosylation of G_i/G_o proteins by PTX under these conditions, the toxin treatment almost completely abolished S1P-induced inhibition of cAMP accumulation (Fig. 2B). This suggests that the S1P-induced ERK activation involves two pathways in terms of PTX sensitivity. When the cells were treated with both PMA and PTX, the S1P action was totally abolished (Fig. 2C). Similarly, the calphostin C-insensitive part of the S1P action was almost completely abolished by PTX treatment (Fig. 2D). These results suggest that the S1P signaling pathway leading to ERK activation is composed of two pathways, i.e., one

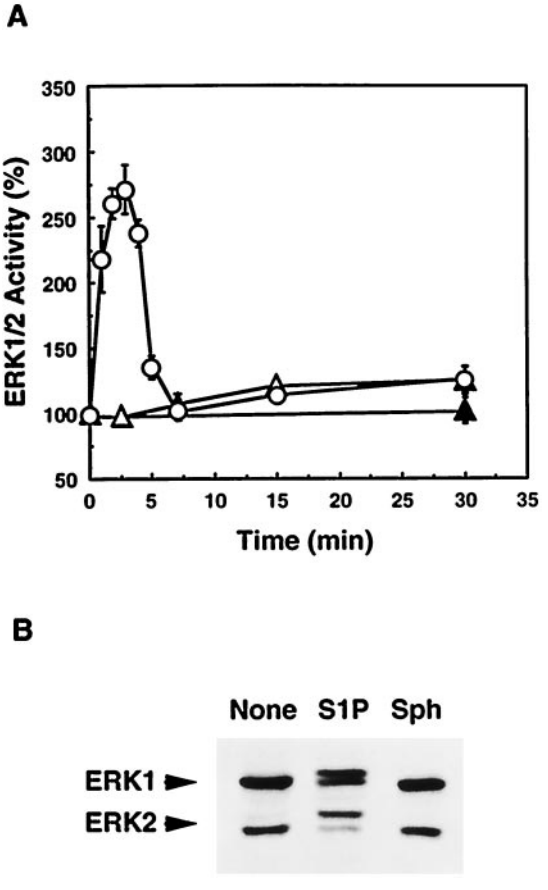

Fig. 1. ERK1/2 activation by S1P in C6 cells. A, time course of ERK1/2 activation. The cells were incubated with 10 μM S1P (○), 10 μM Sph (△) or vehicle (▲) for the time indicated. The lysates were used for measurement of ERK1/2 activity. The results are expressed as percentages of the basal value (147 ± 13 pmol/min/mg) obtained in the absence of these lipids, and the data are shown as the mean ± S. E. of three separate experiments. B, Western blot analysis of ERK1/2. The cells were stimulated with 10 μM S1P, 10 μM Sph, or vehicle for 2.5 min. Data are representative of more than three separate experiments.

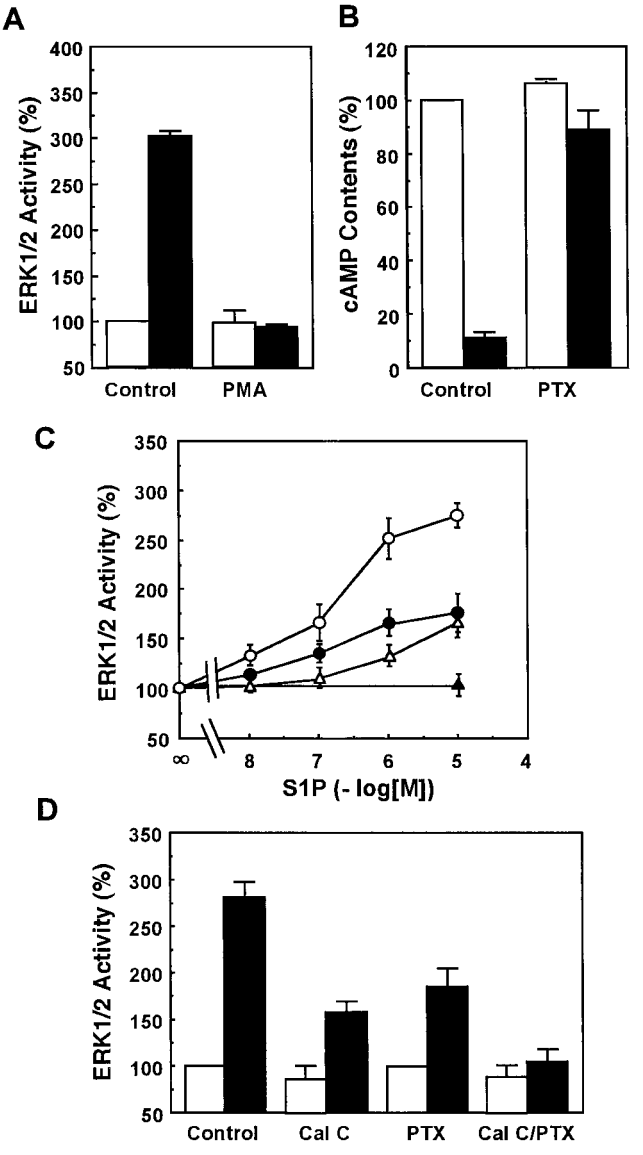

Fig. 2. S1P activates ERK1/2 through two pathways involving either PTX-sensitive G proteins or PKC in C6 cells. A, desensitization of PKC by long-term PMA treatment. The cells were treated with or without 1 μM PMA during a serum deprivation period for 24 h and were then incubated with (filled column) or without (open column) 300 nM PMA for 2.5 min. The lysates were assayed for ERK1/2 activity. The results are expressed as percentages of the basal value (127 ± 13 pmol/min/mg) obtained in the absence of PMA for control cells. B, inhibition of forskolin-induced cAMP accumulation by S1P and its reversal by PTX. The cells were treated with or without 100 ng/ml PTX during a serum deprivation period for 24 h and were then incubated with (filled column) or without (open column) 10 μM S1P in the presence of 10 μM forskolin, 100 μM RO 20-1724 and 0.5 U/ml adenosine deaminase for 5 min. The results are expressed as percentages of the basal value (0.90 ± 0.04 nmol/mg) obtained in the absence of S1P for control cells. C, effect of PMA and/or PTX treatment of the cells on S1P-induced ERK1/2 activation. The cells were treated with PTX and/or PMA for 24 h; vehicle (○), 100 ng/ml PTX (●), 1 μM PMA (△), and a combination of PTX and PMA (▲). The cells were then stimulated with the indicated doses of S1P for 2.5 min. The results are expressed as percentages of the basal value. This basal value without S1P in the control cells was 110 ± 4 pmol/min/mg, and this value was not significantly changed by the treatment with the toxin or the phorbol ester. D, effect of calphostin C, a protein kinase C inhibitor, on S1P-induced ERK1/2 activation. Untreated or PTX-treated cells were preincubated with 1 μM calphostin C for 30 min. The cells were then incubated with (filled column) or without (open column) 10 μM S1P for 2.5 min. The results are expressed as percentages of the basal value (111 ± 43 pmol/min/mg) obtained in the absence of S1P for control cells. All of the data are the mean ± S. E. of three separate experiments.

is the PTX-sensitive and PKC-independent pathway and the other is the toxin-insensitive and PKC-dependent pathway.

Phospholipase C But not Phospholipase D Activation Is Important for PKC-Dependent S1P Signaling. There are at least two ways to produce diacylglycerol for activation of PKC. One mechanism is phospholipase D activation. As shown in Fig. 3A, S1P increased the production of [^{3}H]PA in the absence of ethanol but [^{3}H]PEt decreased in its presence, reflecting the activation of phospholipase D in the [^{3}H]oleic acid-labeled cells. Thus, ethanol switched the product of phospholipase D from PA to metabolism-resistant PEt. However, S1P-induced ERK activation was only slightly (if any) inhibited by ethanol (Fig. 3B). Moreover, this phospholipase D activation was totally sensitive to PTX (data not shown). These results ruled out the possibility of an involvement of phospholipase D in the PTX-insensitive and PKC-dependent signaling pathway. Inability of phospholipase D-derived diacylglycerol to activate PKC has recently been reported in endothelial cells (Pettitt et al., 1997).

Another way to activate PKC is the activation of phospholipase C. S1P has recently been shown to activate phospholipase C and subsequently increase $[Ca^{++}]_i$ in several types

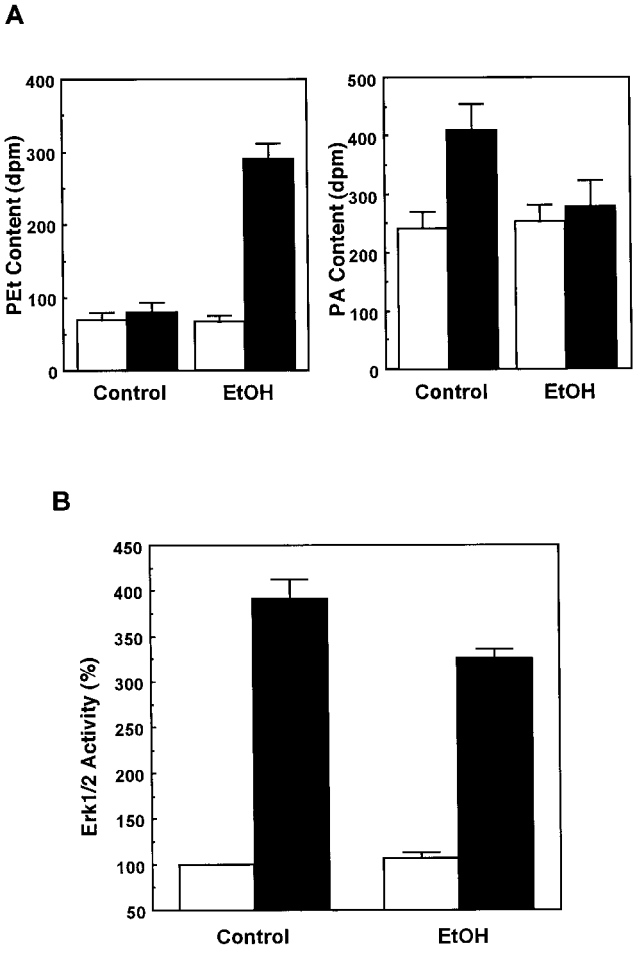

Fig. 3. Phospholipase D activation is not involved in S1P-induced ERK1/2 activation in C6 cells. A, accumulation of PA or PEt by S1P. The cells labeled with [^{3}H]oleic acid were incubated with (filled column) or without (open column) 10 μM S1P in the presence or absence of 2% ethanol (EtOH) for 2.5 min. The results were expressed as radioactivity (dpm) of PA or PEt per radioactivity (100,000 dpm) incorporated into the cellular lipid pool, which was estimated by measurement of the radioactivity in the 5% trichloroacetic acid-precipitated fraction of the cells. Data are the mean ± S.E. of three separate experiments. B, effect of EtOH on S1P-induced ERK1/2 activation. The cells were incubated with (filled column) or without (open column) 10 μM S1P in the presence or absence of 2% EtOH for 2.5 min. The results are expressed as percentages of the basal value (116 ± 11 pmol/min/mg) obtained in the absence of S1P for control cells. Data are the mean ± S.E. of three separate experiments.

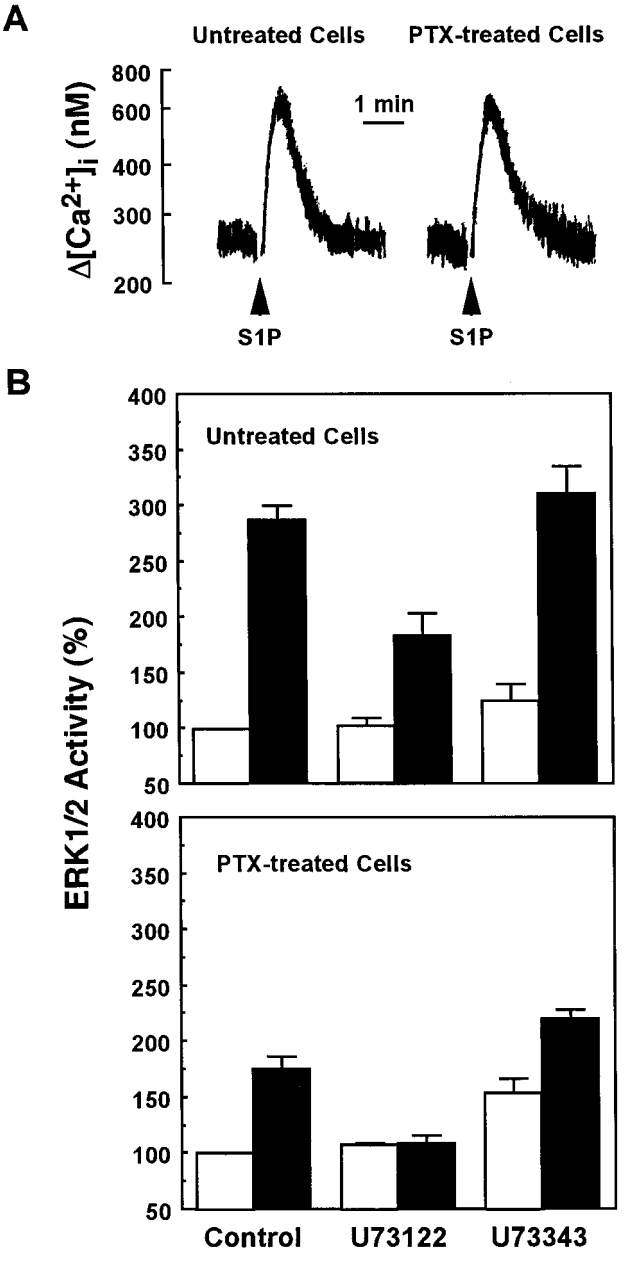

Fig. 4. Involvement of phospholipase C in S1P-induced activation of ERK1/2 in C6 cells. A, effect of PTX on S1P-induced increase in $[Ca^{++}]_i$. The cells were treated with or without 100 ng/ml PTX during a serum deprivation period for 24 h. $[Ca^{++}]_i$ was estimated from the change in the fluorescence of the Fura-2-loaded cells. Data are representative of three separate experiments. The S1P-induced responses (peak value − basal value) were 387 ± 38 nM for control cells and 355 ± 21 nM for PTX-treated cells. B, effect of U73122 on S1P-induced ERK1/2 activation. Untreated or PTX-treated cells were preincubated with either 10 μM U73122, 10 μM U73343, an inactive form of U73122, or vehicle (diemthyl sulfoxide) for 30 min at 37°C. The cells were then incubated with (filled column) or without (open column) 10 μM S1P for 2.5 min. The results are expressed as percentages of the basal value (120 ± 6 pmol/min/mg for untreated cells or 105 ± 6 pmol/min/mg for PTX-treated cells) obtained in the absence of S1P in control cells. Data are the mean ± S.E. of three separate experiments.

of cells (Okajima et al., 1996; Im et al., 1997; Okajima et al., 1997), although PTX sensitivity varied from cell to cell. In C6 cells as well, S1P induced a transient $[Ca^{++}]_i$ increase (Fig. 4A). The increase in $[Ca^{++}]_i$ was inhibited by U73122, a potent phospholipase C inhibitor; the net $[Ca^{++}]_i$ increase (nM) was 329 ± 8 for control cells, 24 ± 7 for U73122-treated cells, and 279 ± 13 for U73343, an inactive U73122 derivative on phospholipase C. This suggests that S1P activated phospholipase C in C6 cells. PTX treatment, however, hardly affected the S1P-induced Ca^{++} mobilization (Fig. 4A). Thus, the phospholipase C activation might account for the PTX-insensitive and PKC-dependent mechanism. Supporting this idea, similar to the calphostin C effect (see Fig. 2D), the phospholipase C inhibitor U73122 partially inhibited the S1P-induced ERK activation in untreated cells but completely inhibited it in PTX-treated cells (Fig. 4B). The inhibitor effect seemed to be related to the inhibitory nature on phospholipase C because the S1P action was hardly affected by U73343, an inactive derivative of U73122, although the derivative alone slightly increased the basal ERK activity by an unknown mechanism(s).

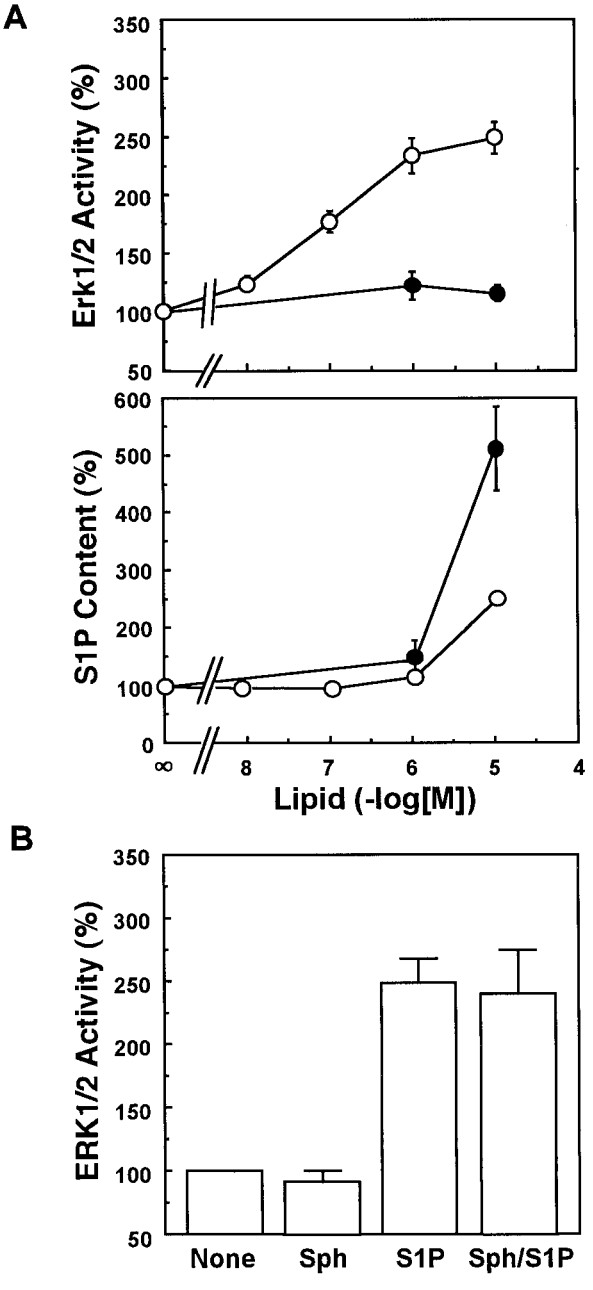

Fig. 5. Intracellular accumulation of S1P is not necessary for exogenously applied S1P-induced ERK1/2 activation in C6 cells. A, effects of S1P and Sph on ERK1/2 activity and intracellular S1P content. The cells were incubated with either S1P (○) or Sph (●) at the indicated concentrations for 2.5 min to measure the ERK activity and intracellular S1P content. For ERK1/2 activity (upper panel), the results are expressed as percentages of the basal value (127 ± 15 pmol/min/mg) obtained in the absence of these lipids, and the data are the mean ± S. E. of three separate experiments. For intracellular S1P content (lower panel), the results are expressed as percentages of the basal value (49 ± 6 pmol/10^6 cells). B, effects of Sph on S1P-induced ERK1/2 activation. The cells were stimulated with 1 μM S1P and/or 10 μM Sph for 2.5 min. The results are the mean ± S.E. of three separate experiments. Results are expressed as percentages of the basal value (128 ± 6 pmol/min/mg) obtained in the absence of these lipids.

Accumulation of Intracellular S1P Is not Necessary for Exogenous S1P-Induced ERK Activation. If S1P-induced ERK activation requires incorporation of the lipid into the cells, intracellular S1P would be expected to increase in response to the exogenous lipid. S1P activated ERK significantly at 10 nM and this activity was saturated at around 1 to 10 μM (Figs. 2C and 5A, top panel). However, a significant increase in intracellular S1P was not detected below 1 μM S1P (Fig. 5A, bottom panel). Moreover, consistent with the results shown in Fig. 1, Sph, a substrate of sphingosine kinase, was ineffective in activating ERK (Fig. 5A, top), whereas this lipid, at 10 μM, increased intracellular S1P more than 5 times (Fig. 5A, bottom). Failure of Sph to activate ERK in spite of an increase in intracellular S1P was not due to its inhibitory nature on PKC (Hannun and Bell, 1987); 1 mM S1P-induced ERK activation was not appreciably affected in the presence of 10 μM Sph (Fig. 5B). These results indicate that an increase in intracellular content in S1P is not necessary for the exogenously applied lipid-induced ERK activation.

Expression of Edg-1 and AGR16/H218 mRNAs in C6 Cells. A couple of cDNAs encoding G protein-coupled receptors have recently been identified as S1P receptors, i.e., Edg-1 (Hla and Maciag, 1990; Lee et al., 1998; Zondag et al., 1998), AGR16/H218 (Okazaki et al., 1993; An et al., 1997), and Edg-3 (Yamaguchi et al., 1996; An et al., 1997). Finally, we examined whether these novel S1P receptor mRNAs were expressed in C6 cells. The reported sizes of these mRNAs were very close, i.e., 3.0 kb for Edg-1 (Hla and Maciag, 1990), 3.1 kb for AGR16/H218 (Okazaki et al., 1993), and 2.8 kb for Edg-3 (Yamaguchi et al., 1996); therefore, the identification of each mRNA should be made with caution. As shown in Fig. 6, both Edg-1 and AGR16/H218 mRNAs were expressed in C6 cells. To confirm whether each band represents the respective mRNA band, we compared the expression patterns of mRNAs in C6 cells with those in CHO cells and Swiss 3T3 fibroblasts. The expression patterns of CHO cells and 3T3 fibroblasts were totally different from those of C6 cells; all of the S1P receptor mRNAs used were expressed in 3T3 fibroblasts, but only AGR16/H218 mRNA was expressed in CHO cells. If the relatively low-density band due to Edg-1 probe in C6 cells reflected AGR16/H218 mRNA, which was abundantly expressed in the cells, we should also detect the band due to the Edg-1 probe in CHO cells which expressed AGR16/H218 mRNA more than C6 cells did. However, this was not the case. Thus, at least two types of S1P receptors may be expressed in C6 cells.

Discussion

PDGF-induced proliferation and ERK activation was partially inhibited by DL-*threo*-dihydrosphingosine, a nonselective sphingosine kinase inhibitor, in association with the inhibition of the growth factor-induced cellular S1P accumulation in 3T3 cells (Olivera and Spiegel, 1993; Rani et al., 1997). Furthermore, exogenously applied S1P activated ERK regardless of the presence of the sphingosine kinase inhibitor in the same cells (Wu et al., 1995; Rani et al., 1997). Based on this inhibitor study, it was proposed that S1P may act intracellularly to activate ERK, as if it were a second messenger (Spiegel and Milstien, 1995). However, this inhibitor experiment is not conclusive evidence showing that S1P acts intracellularly for activation of ERK. It is still possible that S1P acts from outside the cells as an autocrine or paracrine factor; S1P generated by PDGF treatment might be released outside the cells and then interact with cell surface receptors which have recently been identified. Moreover, it has not been demonstrated whether exogenously applied S1P is actually incorporated into the cells to activate ERK. In the most recent S1P studies on ERK regulation (Wu et al., 1995; Pyne et al., 1996; Blakesley et al., 1997; Kozawa et al., 1997; Rani et al., 1997), however, based on this S1P second messenger hypothesis, only the action mechanism as an intracellular signaling molecule appeared to be addressed. In the present study, we analyzed the signaling mechanism underlying exogenous S1P-induced activation of ERK in C6 glioma cells. Exogenous S1P at less than 1 μM activated ERK without a detectable increase in intracellular accumulation of S1P, but sphingosine was ineffective in activating ERK, even though it induced a marked accumulation of intracellular S1P. These results suggest that intracellular accumulation of S1P is not necessary for exogenous S1P-induced activation of ERK and, more importantly, that intracellular S1P is not an essential signal for ERK activation in C6 glioma cells. Thus, at least in C6 cells, S1P activated ERK entirely through extracellular action, possibly through cell surface receptors.

In the present study, the S1P-induced ERK activation was inhibited by PTX treatment of the cells, suggesting the involvement of PTX-sensitive G_i/G_o proteins in the S1P signaling pathway. This further supports the participation of the G_i/G_o protein-coupled receptor in the S1P action. The PTX-sensitive ERK activation by S1P has also been reported in 3T3 fibroblasts (Wu et al., 1995). However, PTX failed to completely inhibit the S1P action in C6 cells, whereas inhibition of S1P-induced cAMP accumulation was almost completely reversed, suggesting that the toxin-insensitive pathway is also involved in the S1P-induced ERK activation. The phospholipase C-PKC system may play a role in the toxin-insensitive pathway (Figs. 2 and 4). In most cases, when the G-protein-coupled receptor is linked to phospholipase C, the PTX-insensitive part of the enzyme activation is mediated through the G_q/G_{11} proteins. Thus, it is reasonable to assume that S1P may stimulate both signaling pathways mediated by G_i/G_o proteins and G_q/G_{11} proteins in C6 cells, although the possibility of S1P as an agonist for the receptor tyrosine kinases is not completely ruled out at the present stage of investigation.

How G_i/G_o proteins and G_q/G_{11} proteins-phospholipase C-PKC regulate the ERK activity in the C6 glioma cells is still uncharacterized; however, the mechanism of ERK activation by G protein-coupled receptors has been extensively investigated in other cell types, especially in the transfected cells with genes coding related signaling molecules and receptors (van Biesen et al., 1995; Gutkind, 1998). In the G_i/G_o protein-mediated pathways, involvement of tyrosine kinases similarly to receptor tyrosine kinases such as the PDGF receptor and epidermal growth factor receptor has also been suggested. In this case, $\beta\gamma$ subunits dissociated from G_i/G_o proteins may play a role in the activation of Src or Src-like tyrosine kinase which phosphorylates Shc and thereby facilitates the recruitment of Grb2 and SOS, resulting in activation of Ras-mediated ERK pathways (van Biesen et al., 1995; Gutkind, 1998). Receptor tyrosine kinases themselves are also targets of phosphorylation to recruit these signaling molecules in the G_i/G_o protein-mediated activation of Ras-ERK pathways (Gutkind, 1998). In the G_q/G_{11} protein-mediated pathways, a $[Ca^{++}]_i$ increase and/or PKC activation through phospholipase C activation may be involved (Gutkind, 1998). In this case, Ras-dependent and independent pathways are proposed. In the former Ras-dependent pathway, the interaction of Src and a novel Ca^{++} and PKC-dependent protein tyrosine kinase, Pyk2, and in the latter Ras-independent pathway, PKC-induced Raf1 phosphorylation may be an important step for the activation of ERK (Gutkind, 1998). In 3T3 fibroblasts, the S1P-induced ERK activation has been shown to involve the activation Raf1/MEK pathway (Wu et al., 1995), and very recent studies suggested the upstream signaling event leading to ERK activation by S1P is the phosphorylation of Crk, a noncatalytic

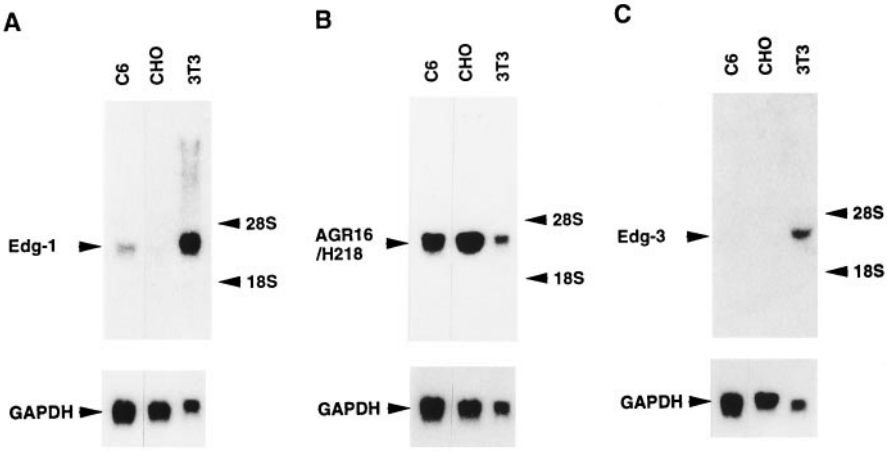

Fig. 6. Northern blot analysis of putative S1P receptors. Total RNAs (10 μg) prepared from C6 cells, CHO cells and Swiss 3T3 fibroblasts were electrophoresed to analyze the expression of mRNAs of Edg-1 (A), AGR16/H218 (B) and Edg-3 (C) as described in *Materials and Methods*. To compare the amount of RNA in each lane, the membrane filters were rehybridized with a probe of GAPDH.

SH2 and SH3 domain-containing adaptor molecule that shares structural homology with Grb2 (Blakesley et al., 1997; Rani et al., 1997). However, an involvement of Ras has not been demonstrated in the S1P-induced ERK activation (Blakesley et al., 1997). In a future study, further characterization of the signaling pathways underlying S1P-induced ERK activation is necessary.

Finally, we detected two types of mRNAs for S1P receptors, i.e., Edg-1 and AGR16/H218, in C6 cells. Thus, these receptors may mediate the S1P-induced ERK activation in this cell line. The receptor characterization in the cells which were transfected with Edg-1 showed that this receptor seems to couple to G_i/G_o proteins resulting in adenylyl cyclase inhibition and ERK activation, but not to the G_q/G_{11} protein-phospholipase C-Ca^{++} signaling pathways (Lee et al., 1998; Zondag et al., 1998). On the other hand, AGR16/H218 appeared to couple to the Ca^{++} signaling pathway (An et al., 1997). We also observed similar results when expression plasmids containing cDNAs of Edg-1 and AGR16/H218 were transfected in CHO cells (K. Sato, N. Murata, J. Kon, and F. Okajima, in preparation). Thus, Edg-1 and AGR16/H218 might represent the receptors which mediate the PTX-sensitive and PKC-independent pathway and the toxin-insensitive and PKC-dependent pathway, respectively. The specific binding of S1P to its cell surface receptor has been reported in platelets (Yatomi et al., 1997), but we failed to demonstrate, in the C6 cells, the specific binding of S1P to its putative receptors due to the very high levels of nonspecific binding. Thus, at the present stage of investigation, there is no direct evidence of the expression of the putative S1P receptor proteins in C6 cell membranes. Nevertheless, our results presented in this study favor the extracellular action mechanism of S1P. These problems could be more clearly resolved by pharmacological experiments using the S1P receptor specific antagonists, but these are not available at present. The recognition of the role of cell surface S1P receptors in the lipid-induced actions will facilitate the exploitation of antagonists and agonists specific to these lipid receptors.

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